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Population Genetics and Morphological Comparisons of Migratory European (*Hirundo rustica rustica*) and Sedentary East-Mediterranean (*Hirundo rustica transitiva*) Barn Swallows

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Abstract

Speciation processes are largely determined by the relative strength of divergent selection versus the magnitude of gene flow. The barn swallow (*Hirundo rustica*) has a broad geographic distribution that encompasses substantial geographic variation in morphology and behavior. The European (*H. r. rustica*) and East-Mediterranean (*H. r. transitiva*) subspecies are closely related, despite differing in morphological and life-history traits. To explore patterns of genetic differentiation and gene flow, we compared morphological and genetic variation among the nonmigratory breeding population of *H. r. transitiva* from Israel and the migratory population of *H. r. rustica* that passes through Israel and compared it with the genetic differentiation between *H. r. transitiva* from Israel and a breeding population of *H. r. rustica* from the United Kingdom that uses a different migratory flyway. Mitochondrial haplotype network analysis suggests that the European and East-Mediterranean populations are intermixed, although there was low but significant genetic differentiation between the subspecies based on both mitochondrial (F_{ST} = 0.025–0.033) and microsatellite (F_{ST} = 0.009–0.014) loci. Coalescent-based analyses suggest recent divergence and substantial gene flow between these populations despite their differences in morphological and behavioral traits. The results suggest that these subspecies are undergoing a differentiation process in the face of gene flow, with selection possibly operating on sexually selected traits.

Key words: *gene flow, genetic differentiation, mate choice, phenotypic divergence, population structure, sexual signal*

Intraspecific geographic variation in phenotypic traits may lead to the evolution of reproductive isolation between populations and hence eventually to speciation (Avise 2000). When populations are connected by gene flow, their level of differentiation is largely determined by the relative strength of divergent selection versus the magnitude of gene flow among them (Mayr 1963; Coyne and Orr 2004). Exploring these dynamics often requires analyses of past and present gene flow derived from molecular markers as well as studies of the underlying causes of differential selection.

The barn swallow (*Hirundo rustica*) is one of the world's most widely distributed passerines and has a broad geographic distribution throughout most of the northern hemisphere. The 6 subspecies (Peters 1960; Cramp 1988; Turner and Rose 1989; Dickinson and Dekker 2001) exhibit substantial geographic variation in morphometric characteristics such as body size and tail streamer length, ventral coloration, and extent of dark breast band (Turner and Rose 1989; Turner 2004, 2006); some of these traits are involved in mate choice (Møller 1988, 1994; Safran and McGraw 2004; Safran et al. 2005; Neuman et al. 2007) and in life-history traits such as migration patterns (Turner and Rose 1989; Turner 2004, 2006).

Recent phylogeographic analyses of the barn swallow (*H. rustica*) subspecies complex based on mitochondrial DNA (mtDNA) and nuclear DNA sequences have suggested

that the European (*H. r. rustica*) and East-Mediterranean (*H. r. transitiva*) subspecies are closely related despite morphological and life-history differences between them (Dor et al. 2010). The subspecies *rustica*, which breeds in Europe, west Asia, and North Africa, has white ventral coloration, long tail streamers, and is a long-distance migrant, whereas the subspecies *transitiva*, which is found in the eastern Mediterranean region, has darker red ventral plumage and slighter shorter streamers compared with *rustica* and is sedentary or makes only short local movements (Turner and Rose 1989; Shirihai 1996; Turner 2004, 2006). Recent evidence suggests that these 2 subspecies might differ in cues used for mate choice: *rustica* females prefer males with long tail streamers (Møller 1988, 1994), whereas in *H. r. transitiva* a mix of both tail streamer length and darker ventral coloration appears to be correlated with male's reproductive success, where darker males with the longest streamers breed earlier and have greater paternity compared with their paler, shorter tailed conspecifics (Vortman et al. 2011). This additional color-based signal of male quality in *transitiva* is similar to that of the American subspecies (*H. r. erythrogaster*), in which males have relatively short tail streamers and females use the males' rufous ventral coloration as their primary mate choice signal (Safran and McGraw 2004; Safran et al. 2005).

The differential use of sexually selected signals by distinct populations may contribute to reproductive isolation in areas where these populations come into contact. This process creates behavioral rather than geographical barriers to reproduction, such as different male song (Irwin et al. 2001) or different breeding phenologies (Friesen et al. 2007). Alternatively, even in the absence of sexual selection, a trait may be retained in one population due to natural selection or due to gene flow. The *H. r. rustica* and *H. r. transitiva* subspecies come into physical contact at least twice a year when *rustica* populations from west Asia and east Europe migrate through the east Mediterranean region to Africa (Figure 1; Turner 2004, 2006). During spring migration (mainly March through May) *H. r. rustica* migrates through the east Mediterranean region when the local *transitiva* are breeding (Shirihai 1996); thus, gene flow is possible between these populations if migrating males mate with local females or if migratory individuals settle to breed in the east Mediterranean.

Here, we explore the morphological differentiation and genetic structure between *H. r. rustica* and *H. r. transitiva* and examine patterns of gene flow between them by comparing the breeding population of *H. r. transitiva* from Israel with the migrating individuals of *H. r. rustica* captured during their transit through Israel. As a partial control for geographic distance between the breeding sites of these populations, we also included comparisons with a breeding population of *H. r. rustica* from the United Kingdom, which migrates across the Strait of Gibraltar rather than through the east Mediterranean region (Figure 1; Wernham et al. 2002). We predict that the genetic differentiation between the *H. r. rustica* and *H. r. transitiva* subspecies will be greater compared with the genetic differentiation among the 2 *H. r. rustica* populations (*H. r. t.* vs. *H. r. r.* Israel or *H. r. r.* UK > *H. r. r.* Israel vs. *H. r. r.* UK). In addition, if there is gene flow between the subspecies facilitated by *H. r. rustica* migrating through breeding *H. r. transitiva*, we predict that the genetic differentiation between *rustica* migrants in Israel and local populations of *H. r. transitiva* should be lower than the genetic distance between the UK *rustica* and the Israeli *H. r. transitiva* (*H. r. t.* vs. *H. r. r.* Israel ≤ *H. r. t.* vs. *H. r. r.* UK).

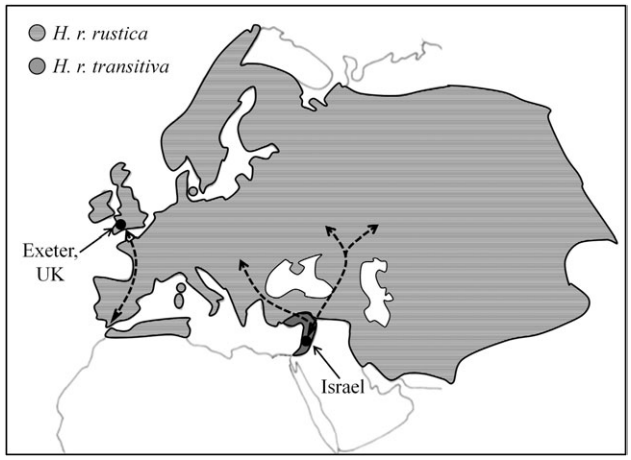

Figure 1. Breeding range distributions and sampling localities (black circles) for migratory *Hirundo rustica rustica* and sedentary *H. r. transitiva*. Dashed arrows represent presumed general migration routes to and from Africa (*H. r. rustica* migrate further into Africa) for the *rustica* population from the United Kingdom (through the Strait of Gibraltar) and for more eastern *rustica* populations from central Europe to western Asia (through the east Mediterranean region).

Materials and Methods

Morphological Measurements

We captured adults from *H. r. transitiva* breeding populations in Israel (n = 136) from 2007 to 2009, *H. r. rustica* adults during their migratory transit through Israel (n = 46) in 2009, and *H. r. rustica* adults from their breeding population in the United Kingdom (n = 194) in 2008 and 2009. We measured tail streamer length for males and females from all 3 populations using a ruler (to the nearest 0.5 mm).

Ventral Coloration Measurement and Analysis

In order to measure ventral coloration, we combined 2 methods: digital photography of whole birds and spectrometry of plucked feathers due to data availability. In order to compare ventral coloration of *H. r. transitiva* and *H. r. rustica* migrants from Israel, we applied digital photography following Stevens et al. (2007), using RAW file formats and manual white balance for 134 *H. r. transitiva* (64 females and 70 males) and 63 *H. r. rustica* migrants from Israel (20 females and 43 males). We used Nikon D-40 digital reflex

camera and adjusted white balance manually for each photograph using a white balance card (WhiBal RAW Ltd) and a Nikon 18% reflectance gray background. For digital image analysis and color scoring, we developed a MATLAB tool (The Mathworks Inc.) that enabled us to exclude pixels with background effect (Vortman et al. 2011). This was done by applying the Max–Lloyd algorithm (Max 1960). Color scoring was done with the sRGB color space, scoring feather's chromatic elements on the R/G and G/B ratio, which is consistent with vertebrates' perception of chromatic properties (Hurvich and Jameson 1957) and with its relative insensitivity to variations in lighting intensity (Kelber et al. 2003). We further simplified our color scoring method and used only the R/B (red/blue) ratio because the variability in green reflectance is negligible in the ventral feathers of the swallows (Vortman et al. 2011). Therefore, we used average R/G ratio from 2 standardized ventral regions (breast and belly, each measured twice) to compare between the 2 populations.

In order to compare ventral coloration of *H. r. transitiva* ($n = 125$; 63 females and 62 males) and *H. r. rustica* from the UK ($n = 149$; 70 females and 79 males), we plucked 3–8 feathers from 2 standardized ventral regions (breast and belly) of each captured bird and mounted them on an index card, stored in the dark, and saved for spectral analysis (Safran and McGraw 2004). The color of feather samples was scored along 3 traditional axes of color (hue, saturation, and brightness) with a reflectance spectrophotometer (Ocean Optics USB4000; range 200–1100 nm; Ocean Optics Inc., Dunedin, FL). This unit quantifies only human visible light that is sufficient for this species because the ventral plumage of barn swallows does not exhibit a unique ultraviolet reflectance peak (Safran and McGraw 2004). Each plumage patch was scored 3 times, and we averaged these scores to determine mean hue, saturation, and brightness for the breast and belly of each swallow. For coloration comparison between populations, we used the average brightness over the 2 ventral regions because it reflects the degree of darkness, which should reflect the difference between the populations.

Due to data availability, we were not able to compare coloration between the 3 populations using the same method of color analyses. However, we were able to measure coloration for 58 individuals of *H. r. transitiva* using both methods and to verify that the results obtained by the 2 methods are correlated ($r^2 = 0.46$, $P < 0.00001$). These methods capture a large part of the within-population variation and are thus well suited for between-population comparisons.

Genetic Sample Collection, DNA Extraction, PCR, Sequencing, and Genotyping

We collected blood samples from 178 adults from *H. r. transitiva* breeding populations in Israel, 71 *H. r. rustica* adults during their migratory transit through Israel, and 62 *H. r. rustica* adults from their breeding population in the United Kingdom (Figure 1). On average, the 2 subspecies can be distinguished morphologically, but the distribution of morphological traits between the subspecies (and between sexes) overlaps, and therefore, it is impossible to rely on morphological characters alone when distinguishing between the subspecies (or determine individual sex). However, the timing of breeding is distinct between these 2 subspecies and therefore affords positive confirmation of subspecies identity. Specifically, the migrating *H. r. rustica* collected in Israel were readily distinguishable from the local *H. r. transitiva* individuals as the *H. r. rustica* samples were collected during February to April 2009 when the local *transitiva* had already initiated breeding and were not spending time at communal roost sites. Moreover, in Israel, the *H. r. rustica* samples were collected from a migratory roosting site with no nearby *H. r. transitiva* breeding sites, and all female *H. r. rustica* captured at those roosts lacked brood patches, whereas at that time of year, brood patches were present in all *H. r. transitiva* females captured elsewhere (Vortman Y, personal observation). To verify gender, we also used genetic methods (see below).

Genomic DNA was obtained using Perfect gDNA Blood Mini kits (Eppendorf, Westbury, NY). We amplified and sequenced 2 mitochondrial protein-coding regions: nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2) and cytochrome b (Cyt-b) for a 30 individuals of each population. To amplify ND2, we used primers METb and TRPc (Hunt et al. 2001), and for Cyt-b, we used primers ProgND5F and ProgCBR (Dor et al. 2010); 10 μl PCR amplifications included 1 μl undiluted DNA, 10 μM Tris–HCl, 50 μM KCl, 3–4 mM $MgCl_2$, 0.25 mM of each nucleotide, 0.25 μM from each primer, and 0.025 U jumpstart *Taq* polymerase (Sigma). PCR amplification conditions were as follows: initial denaturation at 95 °C for 4 min 30 s; 30–35 cycles of denaturating at 95 °C for 1 min, annealing at 54 °C or 62 °C for 1 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 4 min 30 s. PCR products were purified using Exonuclease and Shrimp Alkaline Phosphatase enzymatic reactions (United States Biochemical). Purified reactions were cycle sequenced in both directions using amplification primers and ABI BigDye Terminator. Sequencing products were cleaned using Sephadex columns and processed with an ABI 3730 Automated DNA Analyzer (Applied Biosystems). We aligned forward and reverse strands for each specimen and checked them using Sequencher 4.7 (Gene Codes Corp.). All sequence data were deposited in GenBank (accession no. JN642345–JN642524).

A total of 8 nuclear microsatellite loci previously developed for *H. rustica* or other birds were analyzed (Table 1) for all samples. Forward primers were labeled at the 5′ end with fluorescent tags (PET, 6-FAM, VIC, or NED; Applied Biosystems). Individual PCR amplifications were combined into multiplex mixes to decrease the number of reactions needed per individual (from 8 to 3); 10 μl PCR amplifications included 10–100 ng of genomic DNA, 10 μM Tris–HCl, 50 μM KCl, 1.5–3.25 mM $MgCl_2$, 0.25 mM of each nucleotide, 0.12–0.24 μM from each primer, and 0.025 U jumpstart *Taq* polymerase (Sigma). PCR amplification

Table 1 Marker information for 7 microsatellite loci analyzed for *Hirundo rustica transitiva* and *H. r. rustica* populations

	H. r. transitiva					*H. r. rustica* Israel					*H. r. rustica* UK				
Locus/population	*N*	*K*	H_O	H_E	*P*	*N*	*K*	H_O	H_E	*P*	*N*	*K*	H_O	H_E	*P*
escμ6[a]	178	18	0.854	0.889	0.45	71	17	0.829	0.886	0.09	62	14	0.887	0.885	0.37
ltr6[b]	178	6	0.747	0.742	0.05	71	7	0.743	0.695	0.42	62	5	0.581	0.638	0.20
pocc6[c]	178	17	0.910	0.882	0.90	71	13	0.901	0.897	0.08	62	17	0.903	0.895	0.84
hru6[d]	176	62	0.886	0.963	< 0.01*	68	55	0.941	0.973	0.16	62	50	0.968	0.976	0.22
hir11[e]	176	9	0.756	0.797	0.59	71	12	0.789	0.846	0.01	62	9	0.790	0.804	0.22
hir19[e]	178	15	0.826	0.850	0.17	70	21	0.843	0.899	0.07	62	19	0.742	0.885	0.02
hir20[e]	178	21	0.865	0.869	0.33	71	23	0.831	0.870	0.08	61	19	0.869	0.860	0.32

We describe the number of individuals genotyped (*N*), number of alleles (*K*), observed heterozygosity (H_O), expected heterozygosity (H_E), and *P* values (*P*) for tests for departures from HWE per locus and population. An asterisk indicates significant departure ($\alpha = 0.05$, after sequential Bonferroni correction) from HWE. Footnotes (a)–(e) denote references for primers.

[a] Hanotte et al. (1994).

[b] McDonald and Potts (1994).

[c] Bensch et al. (1997)

[d] Primmer et al. (1995).

[e] Tsyusko et al. (2007).

conditions were as following: initial denaturation at 95 °C for 5 min; 35 cycles of denaturating at 95 °C for 30 s, annealing at 50 °C, 58 °C, or 62 °C for 30 s (specific per multiplex PCR mix), and extension at 72 °C for 30 s; and a final extension at 72 °C for 30 min. PCR products were genotyped on an ABI 3100 Genetic Analyzer (Applied Biosystems) with GeneScan-500 LIZ (Applied Biosystems) as molecular size standard. Allele sizes were estimated using Genemapper v3.7 (Applied Biosystems) and verified and amended by eye to improve the precision of our data.

Birds from all populations were sexed according to the method of Griffiths et al. (1998) through amplification of the chromo-helicase DNA-binding genes using primers P2 and P8 and the standard PCR protocol.

Data Analysis

We used the TCS v1.21 software package (Clement et al. 2000) to infer the parsimony network for the mtDNA sequence data. Networks can give a better representation of the phylogenetic relationship among haplotypes in cases in which sequences are very similar.

For all microsatellite loci, we calculated the number of alleles per locus, observed, and expected heterozygosities using ARLEQUIN v3.11 (Excoffier et al. 2005). We estimated the proportion of null alleles at each locus using Cervus 3.0.3 (Kalinowski et al. 2007). We tested for departures from Hardy–Weinberg equilibrium (HWE) per locus and population and for deviation from linkage equilibrium (LD) for all pairwise locus combinations using GENEPOP v4 (Raymond and Rousset 1995; Rousset 2008). We applied a sequential Bonferroni correction (Rice 1989) to these test results.

We used an Analysis of Molecular Variance (AMOVA) framework implemented in ARLEQUIN v3.11 (Excoffier et al. 2005) to estimate the partition of genetic variation within and among populations using both mtDNA and microsatellite allele frequencies. We grouped the 2 *H. r. rustica* populations for the AMOVA analysis because we were interested in the genetic variation between the *H. r. rustica* and *H. r. transitiva* subspecies. Using ARLEQUIN v3.11, we also calculated pairwise F_{ST} values for mtDNA haplotype frequencies and multilocus pairwise F_{ST} across the microsatellite loci. The *P* value of the test is the proportion of permutations (out of 10 000) leading to a F_{ST} value larger or equal to that observed; 95% confidence intervals for F_{ST} values were calculated using 20 000 bootstraps.

Patterns of population structure were further analyzed for all microsatellite loci using the Bayesian clustering program STRUCTURE v2.2 (Pritchard et al. 2000), which assigns individuals to *K* populations with no prior information on population structure, while maximizing HWE and LD within populations. We ran STRUCTURE for $K = 1$ through $K = 5$, with 20 iterations at each *K* value, using 200 000 generations as burn-in, followed by 2 million generations in a model allowing for admixture and correlated allele frequencies. We examined $\ln P(D)$, the probability of the data given *K*, over the course of the run to ensure that these values stabilized at the end of the burn-in period. We examined the value of $\ln P(D)$ to find the most likely *K* given the data.

In order to estimate divergence times (*t*), migration rates (gene flow; m_1, m_2), and population sizes (θ_1, θ_2, and ancestral θ_A) for these populations, we used the coalescent-based program "Isolation with Migration—analytic" (IMA, Hey and Nielsen 2007). IMA does not assume that mutation, drift and migration are in evolutionary equilibrium, and therefore, it should be more appropriate for estimating parameters for recently diverged populations that may share haplotypes due to either gene flow or ancestral polymorphism. We used IMA only for the 2 *H. r. rustica* and *H. r. transitiva* subspecies population pairs because we found no structure between the 2 *H. r. rustica* populations (see Results). For each analysis, we included data from mtDNA

and 7 microsatellite loci (excluding locus *hir 17*, which showed evidence of null alleles). Initial runs with wide priors were used to identify priors that encompassed the entire distribution of each parameter estimate for use in subsequent runs. Once appropriate priors were identified, we ran each of the 2 run types under the full model (t, θ_1, θ_2, θ_A, m_1, m_2). Each run type was repeated using a different seed number each for longer than 4×10^6 steps to test for consistency. All parameter estimates are scaled to the neutral mutation rate (μ) and are not absolute values.

We then combined the independent "Markov chain Monte Carlo" runs and tested for the fit of the data to simpler demographic models using the nested model approach in the "Load-Trees" mode of IMA. This test calculates log-likelihood ratio statistics for all possible nested models, the significance of which can be assessed using a chi-square test (Hey and Nielsen 2007). Specifically, we tested the full model against models in which either one or both gene flow parameters (m_1, m_2) were set to zero.

One potential bias for the IMA analyses could be an artificially high effective population size of the *H. r. rustica* migrating population. This situation could arise if the *H. r. rustica* migrating population from the eastern side of the migratory divide is structured. However, there was no evidence for structure within the European *H. r. rustica* populations based on a larger study (Santure et al. 2010) and this one (see Results).

The *t*-tests were used to compare pairwise differences in ventral coloration of both males and females between *H. r. transitiva* and *H. r. rustica* from Israel and from the UK and between sexes in each population. One-way ANOVA tests (followed by post hoc Tukey honestly significant difference tests) were used to compare tail streamer length between all 3 populations. Results are presented as mean ± SE unless stated otherwise.

Results

Morphological Characteristics

Comparison of tail streamer length between *H. r. transitiva* and the 2 *H. r. rustica* populations showed that males of migratory *H. r. rustica* from Israel (113.4 ± 1.5 mm) have significantly longer tail streamers than do males of *H. r. rustica* from the UK (104.3 ± 0.8 mm) or *H. r. transitiva* (102.6 ± 1.0 mm) ($F_{2,196} = 18.11$, $P < 0.0001$). However, there was no difference in females' tail streamers between the 3 populations ($F_{2,174} = 0.08$, $P = 0.93$). In all populations, tail streamer length showed sexual dimorphism in which males had longer tail streamers than females (*H. r. transitiva*: $t = 12.13$, degrees of freedom [df] =134, $P < 0.0001$; *H. r. rustica* from Israel: $t = 10.78$, df = 44, $P < 0.0001$; *H. r. rustica* from the UK: $t = 15.74$, df = 192, $P < 0.0001$).

Analysis of ventral coloration from digital photography showed that *H. r. transitiva* are darker than *H. r. rustica* from Israel both for males (R/G ratio: 2.22 ± 0.08 and 1.69 ± 0.05, respectively; $t = 5.07$, df = 111, $P < 0.0001$) and for females (1.79 ± 0.05 and 1.55 ± 0.05, respectively; $t = 2.59$, df = 82, $P = 0.01$). Similarly, analysis of ventral coloration based on spectrometry measures showed that *H. r. transitiva* are darker than *H. r. rustica* from the UK both for males (average brightness: 28.1 ± 0.9 and 40.3 ± 0.8, respectively; $t = 10.06$, df = 139, $P < 0.0001$) and for females (30.8 ± 0.7 and 40.6 ± 0.7, respectively; $t = 10.15$, df = 131, $P < 0.0001$). Ventral coloration was found to be sexually dimorphic for *H. r. transitiva* in which males were darker than females (R/G ratio: $t = 2.33$, df = 123, $P = 0.02$; average brightness: $t = 4.60$, df = 132, $P < 0.0001$) but not for *H. r. rustica* from Israel ($t = 1.77$, df = 61, $P = 0.08$) or *H. r. rustica* from the UK ($t = 0.30$, df = 147, $P = 0.77$).

Molecular Characterization

We aligned the entire Cyt-b gene (1143 bp) and most of the ND2 gene (1023 bp) for 30 individuals from each of the 3 populations. We detected 54 unique haplotypes from 90 individual sequences.

We analyzed a total of 311 samples (178 from *H. r. transitiva*, 71 from *H. r. rustica* from Israel, and 62 from *H. r. rustica* from UK) for 8 microsatellite loci. One locus, *hir 17* (Tsyusko et al. 2007; results not shown) showed evidence of null alleles, and it was therefore excluded from all analyses. The number of alleles detected for the 7 remaining loci ranged from 5 to 62, whereas expected heterozygosities ranged from 0.581 to 0.976 (Table 1). At these loci we found only one deviation from HWE *(hru6* in *H. r. transitiva*) and no significant LD was detected for any pair of loci (after sequential Bonferroni correction). We repeated analyses with and without *hru6*. As the results with and without this locus were similar, we report the results for all loci, including *hru6*.

Phylogenetic Relationships

The parsimony haplotype network (Figure 2) illustrates that these populations are mitochondrially intermixed. We found 54 unique haplotypes from 90 individuals, in which 2 common haplotypes were shared by 13 and 11, respectively, individuals from the 3 populations, 1 haplotype was shared by 1 individual from each of the 2 *H. r. rustica* populations, and 2 haplotypes were shared by 1 or 2 individuals from the *H. r. rustica* from Israel and 1 individual from the *H. r. transitiva* populations. Seven haplotypes were shared by multiple individuals from the same population: 2 from the *H. r. rustica* from Israel population, 2 from the *H. r. rustica* from UK population, and 3 from the *H. r. transitiva* population.

Population Genetic Analysis

AMOVA attributed nearly all the genetic diversity to within-population variation (97.1% from mtDNA haplotypes; 98.9% from microsatellite data; Table 2). Genetic differentiation between the 2 subspecies was low and not significant (differentiation between groups/subspecies: $\Phi_{CT} = 0.0094$, $P = 0.33$, for mtDNA; $\Phi_{CT} = 0.008$, $P = 0.34$, for microsatellites). There was no genetic differentiation between the 2 *rustica* populations based on microsatellite loci (0.3%

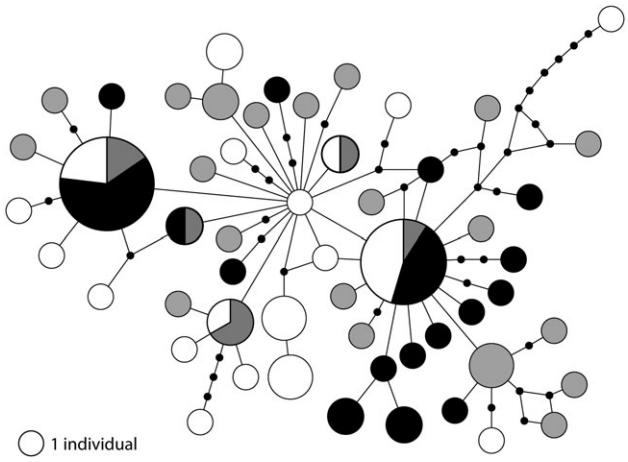

Figure 2. Unrooted parsimony haplotype network as computed using TCS v1.21 for 1) breeding population of the *Hirundo rustica transitiva* from Israel, 2) migrating population of *H. r. rustica* collected in Israel, and 3) breeding population of *H. r. rustica* from UK. Area of circles is proportional to number of individuals with that haplotype. White circles represent *H. r. transitiva* samples, gray circles represent *H. r. rustica* from Israel, and black circles represent *H. r. rustica* from the United Kingdom. Small black circles represent unsampled haplotypes.

of the variance) and weak differentiation (1.97%) based on mtDNA. Conventional F_{ST} showed low (microsatellite data) to moderate (mtDNA sequences) significant differences between *H. r. transitiva* and *H. r. rustica* populations. The 95% confidence intervals for the microsatellite loci F_{ST} values showed that F_{ST} values between *H. r. transitiva* and *H. r. rustica* populations overlap to a large extent (*H. r. transitiva* and *H. r. rustica* from Israel: 0.003–0.017; *H. r. transitiva* and *H. r. rustica* from UK: 0.007–0.027). F_{ST} values based on microsatellite loci showed no differentiation between the 2 *H. r. rustica* populations; however, F_{ST} values based on mtDNA showed a low but significant differentiation between the *H. r. rustica* populations (Table 3). In order to estimate the overall divergence between the subspecies, we calculated F_{ST} values between all *H. r. rustica* samples and *H. r. transitiva* samples. F_{ST} values between the subspecies based on microsatellite loci ($F_{ST} = 0.011$) and mtDNA ($F_{ST} = 0.043$) were statistically significant ($P < 0.001$). In order to explore the possibility that our F_{ST} values were biased by the high number of alleles, we also calculated F_{ST} values without *hru6*, the microsatellite locus with the highest number of alleles (50–62) and found similar F_{ST} values for all comparisons.

Bayesian clustering methods implemented in STRUCTURE were not able to detect the slight differentiation patterns indicated by AMOVA and F_{ST} and instead assigned all individuals from all populations to a single population in the highest probability ($K = 1$: $\ln P(D) = -10793.4$; $K = 2$: $\ln P(D) = -11022.3$; $K = 3$: $\ln P(D) = -10950.4$; $K = 4$: $\ln P(D) = -11000.9$; $K = 5$: $\ln P(D) = -12280.8$).

IMA was applied to estimate gene flow and divergence time between *H. r. rustica* and *H. r. transitiva*. We ran separate IMA analyses for each of the *H. r. rustica* populations (Israel and UK) with the *H. r. transitiva* population. For each of the analyses, the repeated runs with different seed numbers were consistent, demonstrating sufficient sampling of the posterior distribution. We obtained similar estimates for these parameters from the separate analyses for the 2 pairs of populations, representing high levels of gene flow and recent divergence between the 2 subspecies (Table 4). The point estimate for divergence time between the *H. r. rustica* from UK and *H. r. transitiva* was higher and gene flow was lower compared with *H. r. rustica* from Israel and *H. r. transitiva*; however, the 90% credibility intervals of the posterior (90% highest posterior density [HPD]) overlap to a great extent.

We used the nested model approach in IMA in order to test for the fit of the data to simpler demographic models, mainly to examine whether a model that does not include gene flow between the subspecies better explains our data. The full model (t, θ_1, θ_2, θ_A, m_1, m_2) provided the best fit (Table 5). All models that do not include gene flow (either from one population or both) can be rejected (2 log-likelihood ratio [2LLR] > 873, $P < 0.0001$). The other models that include gene flow (either symmetrical or asymmetrical) were significantly worse than the full model as well, although their likelihood values were not as high (2LLR = 6.7–29.4).

Discussion

Recent analysis of the range-wide phylogeography of barn swallows suggested that the European (*H. r. rustica*) and East-Mediterranean (*H. r. transitiva*) populations might be mitochondrially intermixed (Dor et al. 2010) despite strong

Table 2 AMOVA results for *Hirundo rustica transitiva* and *H. r. rustica* populations using mtDNA sequences (ND2, Cyt-b) and 7 microsatellite loci

	mtDNA haplotype frequency					Microsatellite allele frequency				
Source of variation	df	Sum squares	Variance components	% Variation	*P* value	df	Sum squares	Variance components	% Variation	*P* value
Between subspecies	1	0.972	0.005	0.94	0.33	1	12.7	0.024	0.8	0.34
Among population (within subspecies)	1	0.783	0.0099	1.97	0.0007	1	4.3	0.010	0.3	1.00
Within populations	87	42.37	0.487	97.09	<0.0001	619	1840.5	2.973	98.9	<0.0001
Total	89	44.13	0.502			621	1857.5	3.007		

Table 3 Population pairwise F_{ST} values over 7 microsatellite loci (below the diagonal) and over ND2 and Cyt-b sequences (above the diagonal)

	H. r. transitiva	*H. r. rustica* Israel	*H. r. rustica* UK
H. r. transitiva	—	0.025*	0.033*
H. r. rustica Israel	0.009*	—	0.0195*
H. r. rustica UK	0.014*	0.003	—

An asterisk indicates significant F_{ST} values ($\alpha = 0.05$, after sequential Bonferroni correction).

differentiation in morphological and life-history traits. In this study, we examined large sample sizes from 1 population of sedentary *H. r. transitiva* and 2 populations of migratory *H. r. rustica* for morphological differentiation and genetic structure using both mtDNA sequences and microsatellite data to examine the fine-scale genetic structure between these subspecies and to assess levels of gene flow among these populations. From our F_{ST} analyses, we were able to detect fine-scale population structure from both mtDNA and microsatellite loci between the *H. r. transitiva* and the *H. r. rustica* populations but only low differentiation between the 2 *H. r. rustica* populations based solely on mtDNA. Coalescent-based analysis methods (IMA) showed high levels of gene flow between the 2 subspecies following divergence.

The mitochondrial haplotype network showed evidence for intermixture between the 2 subspecies. Two common haplotypes were shared by individuals from the 3 population and 2 more were shared by individuals from *H. r. transitiva* and the *H. r. rustica* from Israel (Figure 2). However, we found significant genetic differentiation between the subspecies. As predicted by our gene flow hypothesis, the genetic distance between *H. r. transitiva* and *H. r. rustica* from UK was slightly greater than the corresponding distance between *H. r. transitiva* and *H. r. rustica* from Israel; however, the confidence intervals for the F_{ST} values overlap extensively. Bayesian assignment analysis (STRUCTURE; Pritchard et al. 2000) did not infer population structure using the microsatellite data. This could be explained by the reduced ability of this method to detect population structure when F_{ST} values are low (Latch et al. 2006).

All analyses indicate that there has been substantial gene flow between *H. r. rustica* and *H. r. transitiva*. A challenge when comparing closely related subspecies is to distinguish between genetic resemblance due to common ancestry or ongoing gene flow. To that aim, we have used the nested model approach in IMA and tested the full model against models in which either one or both gene flow parameters (m_1, m_2) were set to zero. We were able to reject all models of divergence between the 2 subspecies that did not include gene flow. Point estimates for gene flow parameters were relatively higher (and divergence time shorter) between *H. r. transitiva* and *H. r. rustica* from Israel than between *H. r. transitiva* and *H. r. rustica* from UK but the 90% HPD interval for these comparisons overlap to a great extent. The similar patterns for the 2 pairs of populations probably reflect the high admixture of *H. r. rustica* populations. Therefore, the high level of apparent gene flow between *H. r. transitiva* and *H. r. rustica* from UK likely stems from the high level of gene flow within *H. r. rustica* throughout its distribution.

The *H. r. rustica* subspecies has the widest breeding distribution within the barn swallow complex as it extends from Europe and North Africa to west Asia (Turner and Rose 1989; Turner 2004, 2006). The 2 *H. r. rustica* populations sampled in this study are close to the latitudinal extremes of this range as the UK population represents one of the most western populations, whereas the *H. r. rustica* migrants captured in Israel probably derive from more eastern populations from central Europe to western Asia (Russia in the east to Sweden and Italy in the west; Yosef 1997). Despite the widespread extent of our geographic sampling, we were not able to detect any genetic differentiation or structure between those populations. This result is consistent with the recent finding that European breeding populations are not genetically structured based on microsatellite loci (Santure et al. 2010) and is not surprising given the low levels of natal philopatry in migratory populations of this species (Safran 2004; Balbontín et al. 2009).

The 2 genetic markers used in this study—mtDNA and microsatellites—showed varying levels of population genetic differentiation. Analysis based on mtDNA sequences showed higher levels of genetic differentiation than microsatellite loci. The slightly greater differentiation at mtDNA versus microsatellite loci is most likely an outcome of the longer coalescence time of the nuclear-encoded microsatellite loci compared with maternally inherited mtDNA (Zink and Barrowclough 2008). In cases where populations are still connected by gene flow, the effect of the difference in effective population size between the markers is expected to

Table 4 Multilocus maximum likelihood estimates from IMA of divergence times (t), population sizes (θ_1, θ_2, θ_A), and gene flow (m_1, m_2) from the full model with 90% HPD (in parentheses) credibility intervals divergence between *Hirundo rustica rustica* and *H. r. transitiva* populations

Analysis	θ_1[a]	θ_2[a]	θ_A	m_1[b]	m_2[b]	t
H. r. transitiva/*H. r. rustica* from Israel	1.77 (1.77–3785.14)	0.65 (0.65–1144.06)	125.85 (79.76–299.55)	36950 (13350–91050)	34450 (10450–85750)	0.0022 (0.001–0.0085)
H. r. transitiva/*H. r. rustica* from UK	1.77 (1.77–3174.50)	0.80 (0.80–1400.74)	200.00 (47.86–554.78)	21350 (5050–92550)	25350 (4950–81250)	0.0027 (0.0006–0.0101)

All parameter estimates are scaled to the neutral mutation rate (μ).

[a] θ_1 = *H. r. transitiva* and θ_2 = *H. r. rustica* Israel/UK.

[b] m_1 = introgression from *H. r. rustica* Israel/UK to *H. r. transitiva* and m_2 = introgression from *H. r. transitiva* to *H. r. rustica* Israel/UK.

Table 5 Test of nested models of divergence between *Hirundo rustica rustica* and *H. r. transitiva* populations

	H. r. transitiva/*H. r. rustica* from Israel				*H. r. transitiva*/*H. r. rustica* from UK			
Model	Log (*P*)	df	2LLR	*P* value	Log (*P*)	df	2LLR	*P* value
$\theta_1, \theta_2, \theta_A, m_1 = m_2$	−28.52	1	9.03	0.0027	−27.30	1	6.70	0.0096
$\theta_1, \theta_2, \theta_A, m_1, m_2 = 0$	−460.52	1	873.03	<0.0001	−460.52	1	873.15	<0.0001
$\theta_1, \theta_2, \theta_A, m_1 = 0, m_2$	−460.52	1	873.03	<0.0001	−460.52	1	873.15	<0.0001
$\theta_1, \theta_2, \theta_A, m_1 = 0, m_2 = 0$	−460.52	2	873.03	<0.0001	−460.52	2	873.15	<0.0001
$\theta_1 = \theta_2, \theta_A, m_1, m_2$	−34.43	1	20.85	<0.0001	−30.09	1	12.29	0.0005
$\theta_1 = \theta_2 = \theta_A, m_1, m_2$	−38.71	2	29.41	<0.0001	−33.16	2	18.43	<0.0001
$\theta_1 = \theta_2, \theta_A, m_1 = m_2$	−35.32	2	22.63	<0.0001	−31.86	2	15.84	0.0004
$\theta_1 = \theta_2, \theta_A, m_1 = 0, m_2 = 0$	−460.52	3	873.03	<0.0001	−460.52	3	873.15	<0.0001
$\theta_1 = \theta_2 = \theta_A, m_1 = m_2$	−35.36	3	22.71	<0.0001	−33.92	3	19.96	0.0002
$\theta_1 = \theta_2 = \theta_A, m_1 = 0, m_2 = 0$	−460.52	4	873.03	<0.0001	−460.52	4	873.15	<0.0001
$\theta_1 = \theta_A, \theta_2, m_1, m_2$	−29.22	1	10.44	0.0012	−29.05	1	10.21	0.0014
$\theta_1 = \theta_A, \theta_2, m_1 = m_2$	−28.79	2	9.58	0.0083	−33.30	2	18.71	<0.0001
$\theta_1 = \theta_A, \theta_2, m_1 = 0, m_2 = 0$	−460.52	3	873.03	<0.0001	−460.52	3	873.15	<0.0001
$\theta_1, \theta_2 = \theta_A, m_1, m_2$	−36.51	1	25.02	<0.0001	−34.41	1	20.94	<0.0001
$\theta_1, \theta_2 = \theta_A, m_1 = m_2$	−28.89	2	9.78	0.0075	−30.58	2	13.27	0.0013
$\theta_1, \theta_2 = \theta_A, m_1 = 0, m_2 = 0$	−460.52	3	873.03	<0.0001	−460.52	3	873.15	<0.0001

The models are tested against the full model (t, θ_1, θ_2, θ_A, m_1, m_2), by calculating 2LLR.

be less dramatic, but mitochondrial markers would still reach equilibrium quicker than nuclear markers (Crochet 2000).

We examined 2 morphological traits that are known to be important for mate choice preference in barn swallows: tail streamer length and ventral coloration (Møller 1988, 1994; Safran and McGraw 2004; Safran et al. 2005). Whereas tail streamer length did not show a high degree of differentiation between the subspecies (although it was longer for *H. r. rustica* migrants captured in Israel), ventral coloration was significantly darker in *H. r. transitiva* and was not sexually dimorphic in *H. r. rustica* populations. Studies on *H. r. rustica* populations have shown that tail streamer length is heritable (Møller 1991; Saino et al. 2003), whereas ventral coloration was found to be heritable in *H. r. transitiva* population (Vortman Y, unpublished data). These findings, together with the preliminary results for the role of ventral coloration in *H. r. transitiva* mate choice preferences (Vortman et al. 2011), suggest that these traits may be under different selection pressures in the 2 subspecies. However, differences in these traits can be affected also by interactions between genes and the environment, and a more accurate estimation of the genetic basis of these traits should control for environmental contributions.

Our results suggest that the European and East-Mediterranean barn swallow populations have diverged very recently and that there has been gene flow between them following divergence. These populations show very low differentiation in mtDNA sequences and a panel of autosomal microsatellite loci. In contrast, these populations are differentiated morphologically—including in traits that are known to be strongly subject to sexual selection (ventral coloration and tail streamers)—and behaviorally (migration). These contrasting patterns of negligible genetic differentiation and high phenotypic differentiation may represent an incipient differentiation process driven by divergence in signals related to mate choice in the presence of ongoing gene flow. Maintenance of a trait that does not appear to be under sexual selection in one subspecies may be the outcome of this gene flow but could also be maintained by natural selection. Experimental studies should address the relative importance of sexual and natural selection for the maintenance of these traits in the 2 subspecies.

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