Vocal communication in an avian hybrid zone

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Directional hybridization and introgression in an avian contact zone: evidence from genetic markers, morphology and comparisons with lab-raised F1 hybrids

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Manuscript

Contact zones between closely related species are a natural laboratory in which reproductive isolation is put to the test. They can result in hybridization when species isolation is not complete. The genetic characteristics of hybrid individuals and the genetic structure of contact zones give an indication of the stability of these zones, their origin and the level of reproductive isolation between the species. We used mitochondrial DNA, AFLP markers, morphological and colour measurements to investigate a contact zone between two dove species, *Streptopelia vinacea* and *S. capicola*. We also reared F1 hybrids in the lab and compared their features with those of the parental and contact zone populations. The *Streptopelia* contact zone is a hybrid zone characterized by a high frequency of hybrids and a lack of clear parental species forms. There was a high incidence of *S. capicola* mtDNA and an AFLP marker distribution more similar to *S. vinacea* than to *S. capicola*. In both morphology and colour, field hybrids were more similar to *S. vinacea* and significantly different from *S. capicola*. They were also more like *vinacea* than F1 hybrids from the lab, which were genetically truly intermediate and more similar to *S. capicola* in colour. The lab data showed that both types of mixed matings produced viable F1 offspring. Taken together, the results show a unimodal hybrid zone with asymmetric introgression into one parental species: *S. vinacea*. The characteristics of this hybrid zone are uncommon for avian hybrid zones and are most likely due to a combination of geographical and behavioural factors.

Introduction

Secondary contact, between closely related species puts reproductive isolation to the test. Inadequate pre- and/or postmating barriers between the species can result in hybridization. Examining the composition of a contact zone provides insight into the dynamics of contact between the two species (Barton & Hewitt 1985; Price 2008) and may shed light on the causes and consequences of hybridization. Understanding hybridization patterns is important for understanding speciation as hybridization may change the evolutionary direction of one or both taxa (Grant & Grant 1992; Andersson 1999; Seehausen 2004).

Over 200 avian hybrid zones are known (Price 2008) and roughly one in ten species of birds have been known to breed in nature with another species and produce offspring (Grant & Grant 1992). An extensive study of all known hybridization events suggests this figure may be even higher (McCarthy 2006). Studies of avian hybrid zones show a variety of patterns, with different degrees of hybridization (Rohwer & Wood 1998; low: Sætre *et al.* 1999; high: Gee 2004), different fitness consequences (low fitness hybrids: Sætre *et al.* 1999; hybrid superiority within a hybrid zone: Good *et al.* 2000), different levels of introgression (asymmetric: Helbig *et al.* 2001; McDonald *et al.* 2001; none outside the zone: Gee 2004; nuclear, but not mtDNA: Secondi *et al.* 2006). Although several avian hybrid zones have been described, fewer have been examined in sufficient detail to allow an assessment of their dynamics and history (Price 2008). The aim of this paper is to provide such a study.

Hybridization may have various outcomes (Liou & Price 1994; Servedio & Kirkpatrick 1997; Price 2008). If hybrids are inviable, infertile or have reduced fitness, this might generate low frequencies of hybrids and reinforce characters that cause premating isolation. In this case, there would be few mismatches between nuclear and phenotypic characters and low introgression of genes of one species into the other. If hybrid fitness is not greatly reduced compared to parental species individuals, a stable hybrid zone may arise and introgression into parental genomes occur. This would be reflected in the genetic composition of the zone by a large proportion of hybrid individuals and few parental species individuals, combined with introgression from one species to the other. If the contact zone is an ecological transition zone, hybrids might even be best adapted to that zone and give rise to novel adaptations and lineages (Arnold 2006; Price 2008). This bounded hybrid superiority zone would be reflected in high proportions of hybrid individuals and few parental species. There would not be signs of introgression as hybrid genes are selected against outside the zone, where they are less adapted to the ecological conditions.

Exposing the dynamics of a contact zone benefits greatly from an integrated approach that combines nuclear and mitochondrial DNA methods together with phenotypic characters such as morphology, coloration and behaviour (Gaubert *et al.* 2005). Nuclear markers may detect hybrids because of their mixed genomic background. Analyzing widely distributed independent sections of the genome, for instance by using amplified length polymorphism (AFLP) analyses, has proven to be useful in identifying hybrids (Bensch *et al.* 2002) and conclusions from it are less affected by selection on specific genes (Secondi *et al.* 2006). To understand the direction

of hybridization, aspects of sex biased gene flow, mating patterns causing hybridization and backcrossing and possible selection on certain hybrid categories analysis of mitochondrial DNA (mtDNA) is valuable. Mitochondrial DNA is inherited maternally, haploid, shows little recombination and has a smaller effective population size (Babik *et al.* 2003) and will therefore reveal different patterns than nuclear DNA. The levels of mitochondrial and nuclear gene flow may thus differ (Helbig *et al.* 2001; Sætre *et al.* 2001; Babik *et al.* 2003; Helbig *et al.* 2005) and therefore examining both is particularly useful when studying hybrid zones. Measurements of phenotypic traits, such as morphology, colour patterns, or vocalizations may provide additional information on hybridization, introgression and selection of traits. However, such data can be ambiguous. If, for example, hybrids resemble one parental species more than the other with respect to certain phenotypic traits, this might result from directional introgression, but might also result from genetic dominance of that parental species for the trait. Comparing wild-caught hybrids with lab-reared hybrids of known ancestry can help to differentiate between such possibilities and thus contribute to the interpretation of field data. It also allows for a closer examination of the relationship between genetic background and phenotypic traits. In addition, comparing lab-bred hybrids with field hybrids provides insight in the reliability of the genetic markers used, and how accurately, if at all, hybrids can be classified as F1, F2 or backcrosses. So, while most studies focus on either F1 lab characters (Liu *et al.* 1998; Shaw 2000; Congiu *et al.* 2001; Saldamando *et al.* 2005a; Saldamando *et al.* 2005b) *or* natural hybrids (Bensch *et al.* 2002; Albert *et al.* 2006; Secondi *et al.* 2006), combining them can provide a more comprehensive assessment of the patterns of hybridization.

In this study we combine AFLP markers, mitochondrial markers and morphological characters such as tarsus length, wing length and plumage coloration of birds from the field and laboratory-bred F1 hybrids. We examine a narrow contact zone between two African dove species in Uganda: the vinaceous dove, *Streptopelia vinacea* and the ring-necked dove, *S. capicola* (referred to as *vinacea* and *capicola* in the remainder of this paper). These sister species (Johnson *et al.* 2001) are morphologically similar but have very different species specific territorial vocalizations (de Kort *et al.* 2002a). Individuals in the contact zone produce vocalizations intermediate in character between parental species vocalizations (de Kort *et al.* 2002a). Dove vocalizations develop without learning and most likely have a multilocus genetic basis (Lade & Thorpe 1964; Nottebohm & Nottebohm 1971; Baptista 1996; de Kort *et al.* 2002a). Hybridization in doves is known to produce various forms of intermediate vocalizations and hence the results of de Kort *et al.* (2002a) strongly suggest there is hybridization in this contact zone. The aim of this paper is to analyze the likely origin and dynamics of the putative hybrid zone. To this end we (1) describe the differentiation between the two parental species based on analyses of allopatric populations and the contact zone; (2) analyze the composition of the contact zone and compare it with lab-reared F1 hybrids; (3) determine the introgression pattern and whether there is a sex bias in hybrids; and (4) examine the viability of F1 hybrids.

Methods

Population and individual samples

Vinacea and *capicola* are sister species and have a 2.5% mtDNA divergence (Johnson *et al.* 2001). The species specific territorial vocalizations, perch coos, are markedly different and the only discriminating character in the field. These two species meet in a narrow contact zone in Uganda (de Kort *et al.* 2002a; Chapter 3).

The contact zone is found along Lake Albert between the villages of Biiso and Butiaba and is approximately 6 km wide from North to South (from N01º 48' E31º 23' to N01º 45' E31º 23'). We studied adjacent allopatric populations of *capicola* in Queen Elizabeth National Park (N01º46' E31º23'), approximately 270 km south of the contact zone, and *vinacea* in Murchison Falls National Park, south of the village of Paraa and the Victoria Nile (N02º14' E31º34') and approximately 50 km north of this zone. These sites were chosen based on the natural distribution of the species. The species are abundant at these sites. While these populations are close to the contact zone and may have experienced introgression, extensive recordings and observations in these populations provided no evidence of the presence of hybrids when deviation from the species specific vocalizations is taken as a marker for hybridization.

In the contact zone 50 birds were captured and sampled between September 2003 and January 2004 and between September and November 2004; in the *vinacea* population 22 in December 2003; and in the *capicola* population 22 birds between November and December 2003. Of the individuals captured in the allopatric *vinacea* and *capicola* populations, 19 *vinacea* and 18 *capicola* were taken to our lab in Leiden. With these individuals F1 hybrids were bred.

Individuals were trapped using a mist net at water holes or using a mist net in combination with playing back coos in an individual's territory. Blood was taken from the brachial vein (20-50μL) and stored in 500 μL of buffer (0.15 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH=7.5). We used a DNeasy tissue kit (Qiagen) to extract the DNA, following the protocol of the manufacturer for whole nucleated blood.

Sex determination

Birds were sexed using molecular markers and vocalizations (males vocalize, females do so infrequently). The protocol for sexing used Kahn's forward primer (Kahn *et al.* 1998) and Griffiths' reverse primer (Griffiths *et al.* 1998) and is modified from Secondi *et al.* (2002). The PCR was performed in volumes of 10 μl, which included total genomic DNA (9.4 - 4823 nanograms), 0.8 μM of each dNTP, 1x PCR buffer (Qiagen), 0.4 μM of each primer, and 0.5 units *Taq* polymerase (Qiagen). The temperature profile for the PCR was 94 ºC for 2 min followed by 40 cycles of 94 ºC for 10 s, 50 ºC for 10 s and 72 ºC for 30 s, and a final step of 72 ºC for 5 min. 4 μl of the PCR products were run on a 2% agarose gel. Male birds can be identified by a single band on the gel; females by two bands.

AFLP analysis

We used the protocol described in Bensch *et al.* (2002) which was modified from Vos *et al.* (1995) with some slight modifications to customize it to our lab. Total genomic DNA (between 9.4 - 4823 nanograms) was restricted with 2.5 units each of EcoRI (New England Biolabs) and MseI (New England Biolabs), in a total volume of 20 μL containing a 10X ligase buffer (New England Biolabs) and 1μg of BSA. After digestion at 37ºC for 1h, 5 μL of ligation mix was added and incubation continued for another 3h. The ligation mix contained 5.5μM of M-E adaptor (sequences as in Bensch *et al.* 2002) and 0.5 Weiss units of T4 DNA ligase (New England Biolabs, Westburg). The digested DNA with ligated adaptors was diluted 10 times in milli Q H2O and stored at -20ºC.

A preselective amplification was performed in volumes of 20 μL, containing 10 μL of the adaptor-prepared DNA, 0.3 μM of the E-primer with one additional T or A depending on the primer sequence used in the selective amplification, 0.3 μM of the M-primer with an additional C, 0.2 mM dNTPs, 2.5 mM MgCl2, 1x polymerase chain reaction (PCR) buffer and 0.4 units of *Taq* DNA polymerase (Qiagen). The temperature profile for the preselective PCR started with 94 ºC for 2 min. followed by 20 cycles of 94 ºC for 30 s and 72 ºC for 60 s, and a terminal step at 72 ºC for 10 min. The preselective amplification product was diluted 10 times in milli Q H2O and stored at -20 ºC.

The selective PCR was then performed in total volumes of 10 μL, containing 2.5 μL of the diluted preselective PCR product, 0.6 μM each of the E- and M-primer (with three additional bases at the 3'-end, Table 2.1), 0.2 mM dNTPs, 2.5 mM MgCl2, 1x PCR buffer and 0.4 units of *Taq* DNA polymerase (Qiagen). The E-primer was labelled 5' with fluorescein (Fam, Joe or NED). A touch down temperature profile (94 ºC for 2 min followed by 12 cycles of 94 ºC for 30 s, 65 ºC-0.7 ºC/cycle for 30s and 72 ºC for 60s, followed by 23 cycles of 94 ºC for 30 s, 56 ºC for 30 s and 72 ºC for 60 s, and a terminal step at 72 ºC for 10 min) was used to increase the specificity of the amplification. Selective amplification products were separated on a 5% polyacrylamide gel using an ABI Prism 377 automatic sequencer. An internal size standard Rox 500 was used.

We selected 15 - 18 birds from each species (*vinacea* and *capicola* from allopatric populations) to screen for variation with 12 AFLP primer pair combinations. Initial editing and aligning of the gel was done in GENESCAN (Applied Biosystems), after which data was extracted to GENOGRAPHER 1.6.0 (http://hordeum.oscs.montana.edu/genographer) for scoring of bands. Fragments ranging from 100 to 510 base pairs with a fluorescent intensity greater than 100 were scored as present. Bands were scored as dominant markers, giving bands present a value of 1, and bands absent a value of 0. Markers were scored when they were present much more in one species than the other (see below). Some individuals were run twice through the same primer pairs, to check if bands were repeatable between runs. If markers were hard to score or they were not repeatable between gels, they were excluded from the analysis. F1 individuals were checked to make sure the bands they had were present in one of the two parents. If this was not the case the individual was not used for further analysis.

We used two conditions to choose markers for further analyses. Such markers should be at least three times as common in one taxon as in the other, and, be present in at least 4 individuals in the taxon with the highest frequency of that marker. This resulted in 7 primer pair combinations that yielded 46 informative markers (Table 2.1). All individuals were then screened for the presence or absence of these markers.

The software Arlequin 2.0 (Schneider *et al.* 2000) was used to calculate the FST between the two parental populations and the contact zone population. The data was entered as RFLP type haplotype data.

To assess the power of the selected loci to discriminate between both species and hybrid individuals we used the population assignment simulator in AFLPOP version 1.1 (Duchesne

Table 2.1 Frequency of AFLP-derived markers for each of the 7 primer combinations selected. Bold figures indicate which species has the highest frequency of the 'present' allele.

primer combination	marker number	basepair size	*vinacea*	*capicola*	primer combination	marker number	basepair size	*vinacea*	*capicola*
Etga - Mcgg	1	445	0.00	**0.45**	Eaac - Mcta	25	346	0.00	**0.45**
	2	372	0.06	**0.23**		26	286	0.00	**0.36**
	3	319	**0.22**	0.00		27	250	**0.83**	0.09
	4	281	**0.39**	0.00		28	209	0.06	**0.32**
	5	277	0.00	**0.36**		29	206	**0.50**	0.00
						30	147	0.00	**0.36**
Etga - Mcgt	6	466	**0.22**	0.00					
	7	367	0.00	**0.18**	Etcg - Mcga	31	428	**0.28**	0.00
	8	263	**0.39**	0.00		32	419	0.00	**0.23**
	9	257	**0.28**	0.05		33	379	0.06	**0.45**
	10	235	**0.50**	0.05		34	346	0.00	**0.36**
	11	176	**0.50**	0.00		35	312	0.00	**0.23**
						36	273	0.06	**0.32**
Etcg - Mcgt	12	508	**0.28**	0.00		37	250	0.11	**0.73**
	13	452	0.00	**0.18**		38	247	0.22	**0.68**
	14	449	**0.33**	0.05		39	230	0.06	**0.23**
	15	366	0.00	**0.18**		40	158	0.11	**0.86**
	16	227	0.00	**0.23**		41	154	0.00	**0.14**
	17	223	**0.33**	0.05		42	117	0.06	**0.23**
	18	202	0.11	**0.36**					
	19	187	0.06	**0.86**	Etcg - Mcaa	43	422	**0.39**	0.05
	20	184	0.11	**0.64**		44	290	0.06	**0.45**
	21	130	**0.78**	0.00		45	281	**0.50**	0.00
						46	146	**0.39**	0.05
Etga - Mcga	22	405	0.06	**0.27**					
	23	363	0.00	**0.23**					
	24	305	**0.28**	0.05	Total			18	28

& Bernatchez 2002) as done in Albert *et al.* (2006). Based on the allelic frequencies observed in *vinacea* and *capicola* populations, the AFLPOP simulator randomly generated 1000 genotypes of each of the six following categories: pure *vinacea*, pure *capicola*, first generation hybrids (F1), backcrosses to *vinacea* and *capicola* (BCv and BCc) and second generation hybrids (F2). Those 6000 simulated individuals were then reassigned to their most probable category. Since the probability of erroneous assignment between the F1, BCv, BCc, and F2 hybrid categories was high (see Results), we combined these into a single category of hybrids (FN).

With AFLPOP we then assigned 22 *capicola*, 18 *vinacea*, 50 contact zone individuals and 33 F1 hybrids to hybrid or parental genotypes. For individuals of unknown origin, this software computes the log-likelihood of possessing parental or hybrid genotypes (first- or second-generation hybrids or backcrosses) based on allele frequencies estimated from known allopatric populations. *Vinacea* and *capicola* were entered as the 'source populations'. Individuals from all populations were entered as 'to be allocated', to get a likelihood score for each individual. We set the analysis parameters as follows: zero replacement value=0.001, number of artificial genotypes=1000, P=0.001, and minimal likelihood difference=0 (comparable to the simulations). Pure genotypes will be identified by a large difference in the calculated likelihood of origin. Hybrids will have similar likelihood scores for the two taxa and therefore a small likelihood difference, due to their mixed genetic makeup.

Mitochondrial DNA analysis

22 *vinacea*, 22 *capicola*, 50 contact zone individuals and 40 F1 individuals were classified according to their mitochondrial haplotype. In GenBank there are mitochondrial sequences for one *vinacea* individual and one *capicola* individual from far allopatric populations: Central African Republic for *vinacea* and South Africa for *capicola* (see Appendix, Table A2.1 for accession numbers). The sequences of three mitochondrial genes, cytochrome b (cyt b), NADH dehydrogenase subunit 2 (ND2), and cytochrome oxidase I (COI), were aligned using Bioedit (Hall 1999). We looked for differences between the sequences and for restriction enzymes that could cut at those sites. We then chose one site within each gene for cutting. PCR primer pairs that would amplify segments (200 bp) of these genes in which the restriction site was found were designed using the Primer3 program available online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The base pair numbers of the restriction sites were determined after alignment of the three genes with the corresponding Chicken genes (GenBank Accession number NC_007236).

PCR reactions were performed in volumes of 12.5 µl, which included total genomic DNA (9.4 - 4823 nanograms), 1.25 µM of each dNTP, 0.4 µM of each primer, 1.25 units of *Taq* DNA polymerase (Qiagen) and 1x Qiagen PCR buffer. Initial denaturing at 94 °C for 4 min. was followed by 40 cycles of 94 °C for 15s, 53 °C for 30s and 72 °C for 30s, and a terminal step of 72 °C for 5 min. Restriction enzymes (Appendix, Table A2.1) were used to digest the PCR products, following the manufacturer's instructions (New England BioLabs). The PCR products were checked on a 2% agarose gel for the presence and size of one (*capicola*) or two (*vinacea*) bands at all three loci.

Each individual was classified as a *vinacea* (v) or a *capicola* (c) at each site, with the genes scored in the following order: ND2, COI, cyt b. We will refer to the outcome for each individual as its haplotype 'profile'. Individuals were expected to be either 'vvv' or 'ccc'. However, two *vinacea* individuals had a 'vcc' profile, one had a 'ccc' profile, and one had a 'vcv' profile (Figure 2.3). Three *capicola* individuals had a 'vcc' profile. Of these samples (4 *vinacea*, 3 *capicola*) the whole 200 bp segments were sequenced (Macrogen) that included the restriction site for the COI and cyt b genes. Two samples of each species that showed a consistent profile across the three restriction sites ('vvv' or 'ccc') were also sequenced as a control.

The sequences were edited with Sequencher (Genecodes, Madison,WI), checked for stop codons and aligned with the known sequences from GenBank to be sure no Numts (mitochondrial insertions in nuclear DNA) had been sequenced. After alignment with Bioedit the differences between the sequences were compared with the results obtained with the restriction enzyme method to check if our restriction method was reliable in assigning individuals to certain haplotypes.

Morphological and colour measurements

We measured bill length (from the tip of the bill to the start of the feathering) and tarsus length (when gently bent at right angles both at the intertarsal joint and at the joint with the foot, from just above the intertarsal joint to just below the foot joint) using dial callipers (mm). Contact zone individuals (n=49) were measured in the field, *vinacea* (n=14), *capicola* (n=15) and F1 individuals that had reached adulthood (n=30) were measured in the lab.

Capicola individuals (n=13) were photographed both in the field and in the lab. Contact zone individuals (n=44) were photographed in the field. *Vinacea* (n=11) and F1 individuals (n=11) were photographed in the lab. Photographs were made with a digital camera (Fuji Finepix S300). A reference colour card (Kodak colour control patches, Eastman Kodak company, 1997) was held next to the bird when it was photographed to be able to standardize photographs taken under varying light conditions. Several photographs were taken of each individual (varying from 2 to 6).

Photographs were measured in Adobe Photoshop 6.0. The white patch on the reference card was used to standardize the photographs. The colour sample tool was then used to measure RGB-colours (red, green, blue). The colour sample tool took an average measure for a square of 5x5 pixels. Four squares were measured both on the head and chest of an individual. These measures were then averaged to have one head and one chest measure per individual.

The groups were tested for equality of variances for each measure. Five colour measures had unequal variances and therefore the Welch statistic was used to test for equality of means between groups and a Games-Howell post hoc test was used for pair wise comparisons between the groups. For measures with equal variances an ANOVA with Tukey HSD post hoc was used. SPSS 12.0.1 was used for the statistics.

F1 viability

When breeding F1 individuals we kept track of the eggs laid, hatched and surviving young to get an idea of fertility and viability of F1 individuals. The breeding program was not specifically designed to gather such data in a systematic way, but the results give, at least, a tentative indication of F1 viability. With a binomial generalized linear model with the logit link function in R software version 2.4.0 (Ihaka & Gentleman 1996) we analyzed the effect of cross type on fertility, hatchability and survival. We checked for over dispersion and adjusted for it if necessary using a quasi-likelihood approach. Using F-tests we analyzed whether the effect of cross type was significant for each variable.

Permits

Individuals were caught and sampled with permission from the Uganda Wildlife Authority (permit no. 00455) and the Uganda National Council for Science and Technology (permit no. EC 578). Blood samples and live birds were exported with permission from the Uganda Wildlife Authority (Material Transfer Agreement no. 028 and no. 0041, License to export scientific material serial numbers 47816 and 47832 and License to export live, non-scheduled animals serial no. 2505), the Uganda National Council for Science and Technology and the CITES Authority (permit numbers 001477 and 001684) in Uganda. Blood samples and live birds were imported into the Netherlands with permission from the Voedsel en Waren Autoriteit (TRVV/52167, TRVV/52190). Blood samples taken from captive animals were approved by the Leiden University committee for animal experiments, license number: DEC05065.

Results

Population differences and individual assignment

All population pair wise distances were significant. The FST between *vinacea* and *capicola* is 0.37, *vinacea* and the contact zone 0.13, and *capicola* and the contact zone 0.21.

Based on the simulation and reassignment procedures performed with AFLPOP using the six dove categories (*vinacea*, *capicola*, F1, BCv, BCc, and F2), the assignment success for pure *vinacea* and pure *capicola* was 94.5% and 95.6%. The misassignment probabilities for F1, BCv, BCc, and F2 were quite high: 61.1%, 39%, 38.9%, and 67.6% respectively. In order to reduce misassignment these four categories were pooled into a hybrid category (FN), which resulted in a misassignment rate of 1.7%, 14.9%, 15.4%, and 2.3% for the F1, BCv, BCc, and F2 respectively (misassignment here is not being assigned to the pooled FN category). The overall misassignment rate was then 7.4%.

Based on AFLP, *capicola* individuals were all classified correctly to *capicola*, except one which was classified as a backcross to *capicola*. The *vinacea* individuals were all classified correctly to *vinacea*, except for one, which was classified as a backcross to *vinacea*. The correct assignment of F1 individuals to the F1 category was 27.3%. This low percentage was expected from the simulation

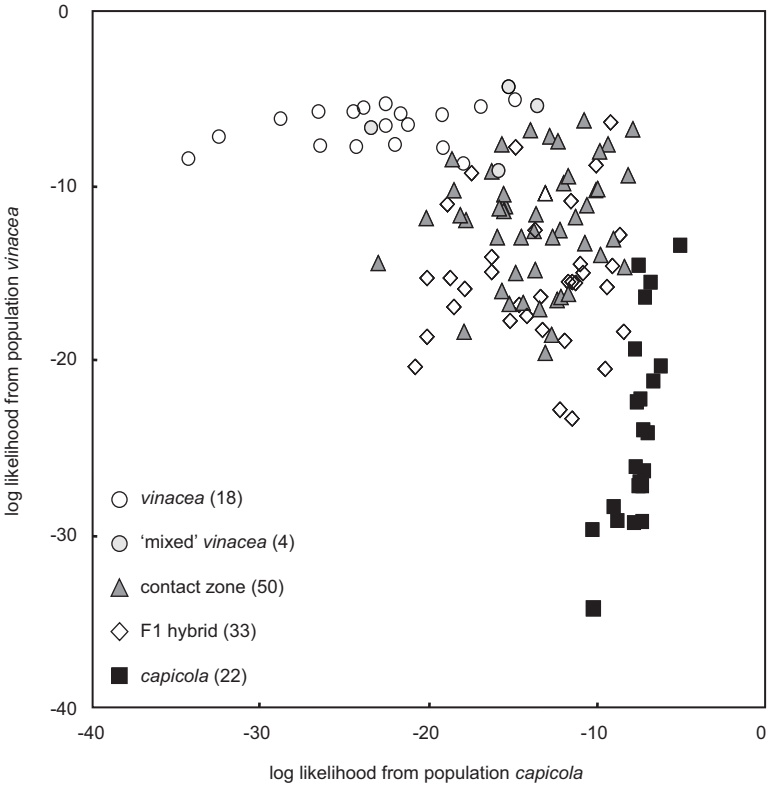


Figure 2.1 Result of likelihood calculations with AFLPOP 1.1. Parental species individuals, *vinacea* and *capicola* are shown, as well as individuals from the contact zone and F1 hybrids bred in the lab. The likelihood of belonging to the *vinacea* population is plotted against the likelihood of belonging to the *capicola* population. *Vinacea* and *capicola* cluster separately and do not overlap. The diagonal is an area of equal likelihood of belonging to one population or the other and it is where we would expect to find hybrids. In this area between the two species clusters, the individuals from the contact zone overlap with the F1 hybrids. Individuals labelled 'mixed' *vinacea* are individuals from the *vinacea* population but had *capicola* mitochondrial DNA.

data. When hybrid categories were taken together, the correct assignment of F1 individuals to the hybrid category was 93.9% and of individuals from the contact zone to the hybrid category was 94% (three individuals were classified to *vinacea*). Individuals from the contact zone fall in between the clusters of the two parental species with similar likelihoods of belonging to both species, and they overlap with F1 individuals, indicating that individuals in the contact zone are hybrids (Figure 2.1). The distribution of likelihood differences for each population (Figure 2.2) suggests contact zone birds seem to be slightly more *vinacea*, and that the hybrid population may include backcrosses to *vinacea*. We used likelihood methods for our analysis, but the results were similar when using the Bayesian method of the software NewHybrids (Anderson & Thompson 2002, results not shown).

Table 2.2 Comparison of cytochrome oxidase I and cytochrome b sequences for *capicola* and *vinacea*. Gray squares are typical *capicola* nucleotides and white squares are typical *vinacea* nucleotides (with the exception of the A nucleotide at position 52). Empty squares indicate individuals were not sequenced at that gene. An * indicates the restriction site.

		COI							cyt b					
individual	profile	52*	103	133	160	182	214	220	54	69	70*	114	163	166
V4	vcv	T	A	C	A	C	T	A	C	T	C	C	T	C
V5	ccc	T	A	T	G	C	T	G	T	C	T	T	T	T
V10	vcc	T	A	T	G	C	T	G	T	C	T	T	T	T
V14	vcc	A	A	T	G	C	T	G	T	C	T	T	T	T
C2	vcc	T	A	T	G	C	T	A	T	C	T	T	T	T
C4	vcc	T	A	T	G	C	T	A	T	C	T	T	T	T
C8	vcc	T	A	T	G	C	T	G	T	C	T	T	T	T
V6	vvv								C	T	C	C	C	C
V7	vvv								C	T	C	C	C	C
V8	vvv	C	G	C	A	T	C	A	C	C	C	C	C	C
V12	vvv	C	G	C	A	C	C	A	C	T	C	C	C	C
V16	vvv								T	T	C	C	C	C
V17	vvv								C	T	C	C	C	C
C10	ccc	T	A	T	G	C	C	G	T	C	T	T	T	T
C12	ccc	T	A	T	G	C	T	G	T	C	T	T	T	T
C14	ccc								T	C	T	T	T	T
C15	ccc								T	C	T	T	T	T
C20	ccc								T	C	T	T	T	T
C21	ccc								T	C	T	T	T	T
GenBank *vinacea*		C	G	C	A	T	C	A	C	T	C	C	C	C
GenBank *capicola*		T	A	T	G	C	T	G	T	C	T	T	T	T

Mitochondrial DNA

Eighteen *vinacea* individuals were 'vvv', 2 were 'vcc', 1 was 'vcv' and 1 was 'ccc'. Nineteen *capicola* individuals were 'ccc' and 3 were 'vcc'. Six contact zone individuals were 'vvv', 25 were 'ccc', 18 were 'vcc' and 1 was 'vcv' (Figure 2.3). All F1 individuals showed the same profile as their mothers.

Vinacea individual V5 showed a 'ccc' profile and V10 and V14 showed 'vcc' profiles. All these individuals showed *capicola* sequences for both sequenced genes (COI and cyt b). The *vinacea* individual that showed a 'vcv' profile, was V4. When we sequenced a segment of the COI gene, it had 4 *vinacea* nucleotides, the rest were *capicola*. At the cyt b gene, it had a *vinacea* sequence, except for one *capicola* - like nucleotide (Table 2.2). Three *capicola* individuals, C2, C4 and C8 showed a 'vcc' profile. Both C2 and C4 showed complete *capicola* sequence for the sequenced sections of

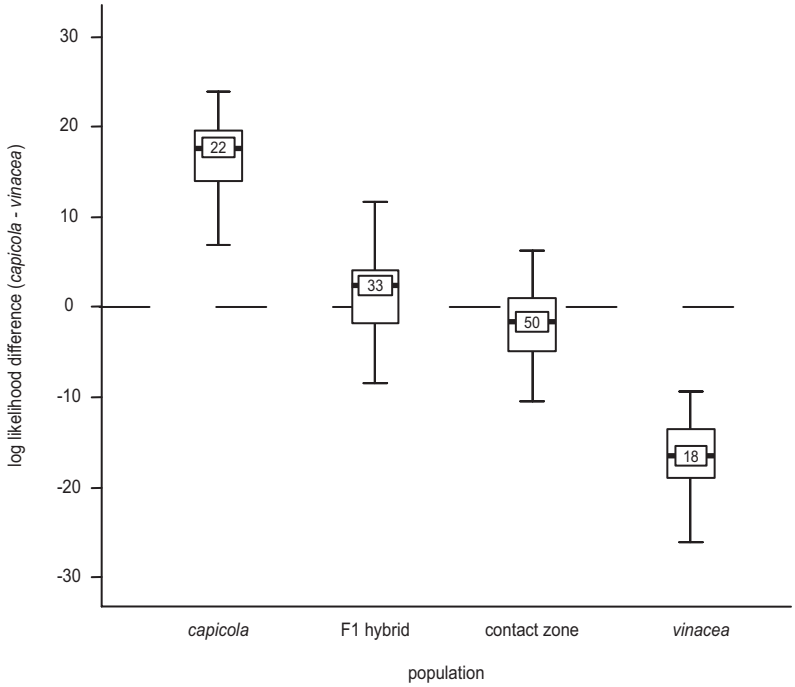


Figure 2.2 Difference in the log likelihood of belonging to *capicola* or *vinacea* populations (*capicola* – *vinacea*) for each 'population' based on likelihood results from AFLPOP. A positive value indicates a high likelihood to belong to *capicola*, while a negative value indicates a high likelihood to belong to *vinacea*. For both the contact zone population and F1s the likelihood differences are close to zero, indicating they are hybrids. Contact zone individuals and F1s differ in their likelihood differences distribution. Contact zone individuals have a likelihood that is slightly more towards *vinacea*. Box plots represent the median, interquartile range and full ranges. Numbers in the box plots show the number of individuals in each sample.

both genes, except for one *vinacea* - like base in the COI sequence. C8 showed a complete *capicola* sequence for both genes.

This confirms that our restriction method was accurate in classifying individuals' haplotypes. Haplotypes classified as 'vcc' are *capicola* haplotypes. Individuals with a 'ccc' profile indeed had complete *capicola* sequences. *Vinacea* showed introgression of *capicola* haplotypes (3 individuals). Most individuals from the contact zone had *capicola* haplotypes. One individual had a 'mixed' haplotype like the *vinacea* individual V4. The 4 *vinacea* individuals whose profile was not 'vvv' (V4, V5, V10 and V14) were not used as allopatric parental species individuals to calculate the likelihoods of assignment. They were entered to be classified as either parental species or hybrids and 3 of them were assigned to *vinacea* with the highest likelihood. One was classified as a backcross to *vinacea*. So, *capicola* haplotypes dominate in the hybrid zone, with some evidence of their introgression in the *vinacea* population.

Morphology

Birds with the ambiguous mtDNA profiles were excluded in the morphological and colour analyses. The bill lengths differed between the 4 groups ($F_{3, 104}$=4.364, *P*=0.006, Figure 2.4 and in

the Appendix, Table A2.2). *Vinacea* has a shorter bill than *capicola* (Tukey's post hoc test, P=0.026) with those of contact zone birds and F1s in between. The tarsus lengths differed between the 4 groups ($F_{3, 105}$=9.126, P<0.001, Figure 2.4 and in the Appendix, Table A2.2). *Capicola* had a significantly longer tarsus than *vinacea* (Tukey's post hoc test, P<0.001), F1 hybrids (P=0.023) and wild hybrids (P<0.001). F1 hybrids and contact zone individuals were intermediate in tarsus length, and did not differ from each other. F1 hybrids also had a significantly longer tarsus than *vinacea* (P=0.038) while contact zone individuals did not differ in this respect from *vinacea,* suggesting that contact zone individuals are more *vinacea*-like than F1s.

Differences between the sexes within each group were also analyzed. *Vinacea* males (n=9) had a larger tarsus than females (n=5) (t_{12}=2.949, P=0.012). *Capicola* males (n=8) also had a larger tarsus than females (n=8) (t_{14}=3.201, P=0.006). Contact zone males (n=32) also had a larger tarsus than females (n=17) (t_{47}=2.288, P=0.027). In the F1 hybrids females (n=15) had a longer bill than males (n=15) (t_{28}=-3.362, P=0.002). See Appendix, Table A2.3 for all tests and their P values.

Differences in bill length and tarsus length of F1 hybrids was also analyzed depending on the composition of the parental individuals crossed to produce F1 hybrids. There were no

Table 2.3 Pairwise comparisons of *capicola*, *vinacea*, contact zone and F1 hybrid populations for 6 colour measures. Pairwise comparisons were carried out with Games-Howell post hoc test. *The pairwise comparisons for the chest blue parameter were carried out with a Tukey HSD post hoc test. See Results. Bold values indicate significant P values.

parameter	populations compared	P	parameter	populations compared	P
head			chest		
red	*capicola* - *vinacea*	**<0.001**	red	*capicola* - *vinacea*	**<0.001**
	capicola - F1 hybrid	0.862		*capicola* - F1 hybrid	0.989
	vinacea - F1 hybrid	**<0.001**		*vinacea* - F1 hybrid	**<0.001**
	capicola - contact zone	**<0.001**		*capicola* - contact zone	**<0.001**
	vinacea - contact zone	0.949		*vinacea* - contact zone	0.481
	F1 hybrid - contact zone	**0.001**		F1 hybrid - contact zone	**<0.001**
green	*capicola* - *vinacea*	**0.001**	green	*capicola* - *vinacea*	**<0.001**
	capicola - F1 hybrid	0.439		*capicola* - F1 hybrid	0.793
	vinacea - F1 hybrid	**<0.001**		*vinacea* - F1 hybrid	**<0.001**
	capicola - contact zone	**<0.001**		*capicola* - contact zone	**0.002**
	vinacea - contact zone	0.571		*vinacea* - contact zone	**0.004**
	F1 hybrid - contact zone	**<0.001**		F1 hybrid - contact zone	**0.001**
blue	*capicola* - *vinacea*	**0.031**	blue*	*capicola* - *vinacea*	**0.001**
	capicola - F1 hybrid	0.181		*capicola* - F1 hybrid	0.972
	vinacea - F1 hybrid	**<0.001**		*vinacea* - F1 hybrid	**<0.001**
	capicola - contact zone	**<0.001**		*capicola* - contact zone	**<0.001**
	vinacea - contact zone	**0.001**		*vinacea* - contact zone	0.980
	F1 hybrid - contact zone	**<0.001**		F1 hybrid - contact zone	**<0.001**

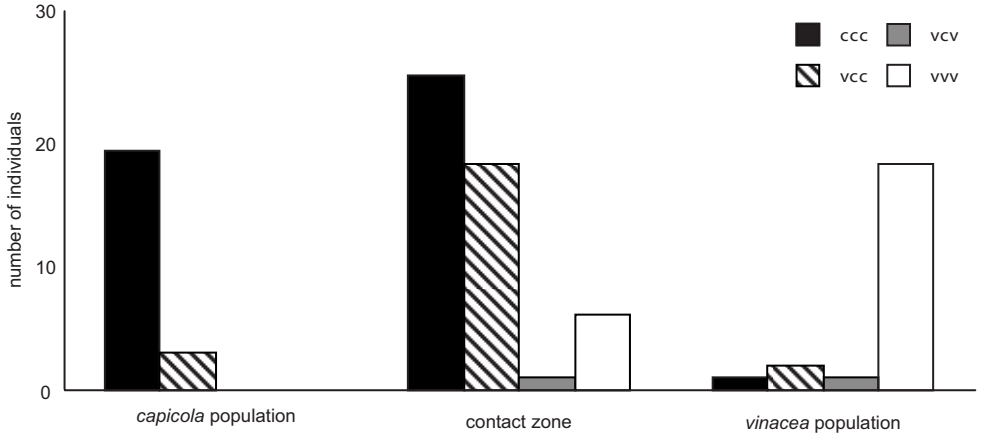


Figure 2.3 Haplotype profile distribution in the three populations studied. Each individual was classified as a *vinacea* (v) or a *capicola* (c) at three restriction sites, one in each gene: ND2, COI, cyt b, scored in that order. *Vinacea* haplotypes are 'vvv' and *capicola* haplotypes are 'ccc' and 'vcc'. Two individuals with a 'vcv' haplotype, seem to have mixed haplotypes. Please see Methods and Results for more details.

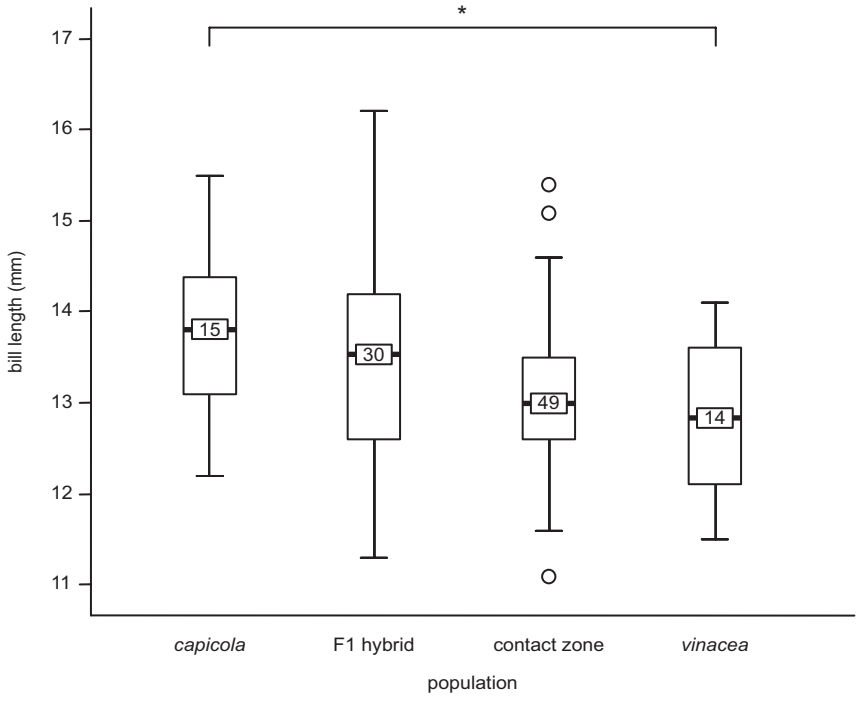


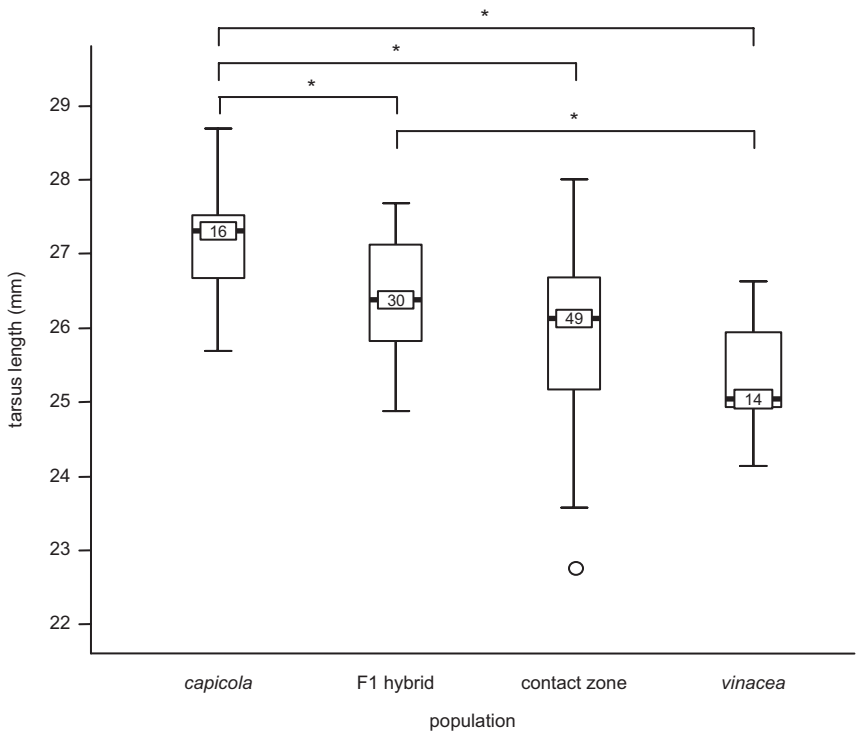
Figure 2.4 Bill length and tarsus length (opposite page) for *capicola*, F1 hybrids, contact zone and *vinacea* populations. *Vinacea* and *capicola* are significantly different from each other. F1 hybrids and individuals from the contact zone are intermediate and do not differ from each other. However, contact zone individuals do not differ significantly from *vinacea*.

differences between individuals from a cross of a *capicola* father and a *vinacea* mother (n=15) and a cross with a *vinacea* father and a *capicola* mother (n=15) in bill length (t_{28}=-0.608, *P*=0.548) and tarsus length (t_{28}=-1.747, *P*=0.092). See Appendix, Table A2.4 for all tests and their *P* values.

Colour

Capicola photos taken from individuals in the lab and the field were compared with a paired samples T test. For all but one measure they were found to not be significantly different. The five measures that were not significantly different were: Head Red (T_{12}=2.095, *P*=0.058), Head Green (T_{12}=-1.004, *P*=0.335), Chest Red (T_{12}=-1.343, *P*=0.204) and Chest Green (T_{12}=-0.049, *P*=0.962) and Chest Blue (T_{12}=-0.103, *P*=0.920). Head Blue was significantly different between the lab and field photos (T_{12}=-3.771, *P*=0.003). We therefore found it reasonable to compare *capicola*, *vinacea* and F1 lab photos with contact zone individuals photographed in the field.

All 6 colour measures differed between the 4 populations: Head Red (Welch statistic$_{3,27.905}$=33.360, *P*<0.001), Head Green (Welch statistic$_{3,29.719}$=29.350, *P*<0.001), Head Blue (Welch statistic$_{3,31.338}$=26.804, *P*<0.001), Chest Red (Welch statistic$_{3,29.117}$=38.533, *P*<0.001) and Chest Green (Welch statistic$_{3,27.315}$=25.533, *P*<0.001) and Chest Blue ($F_{3,75}$=15.736, *P*<0.001).



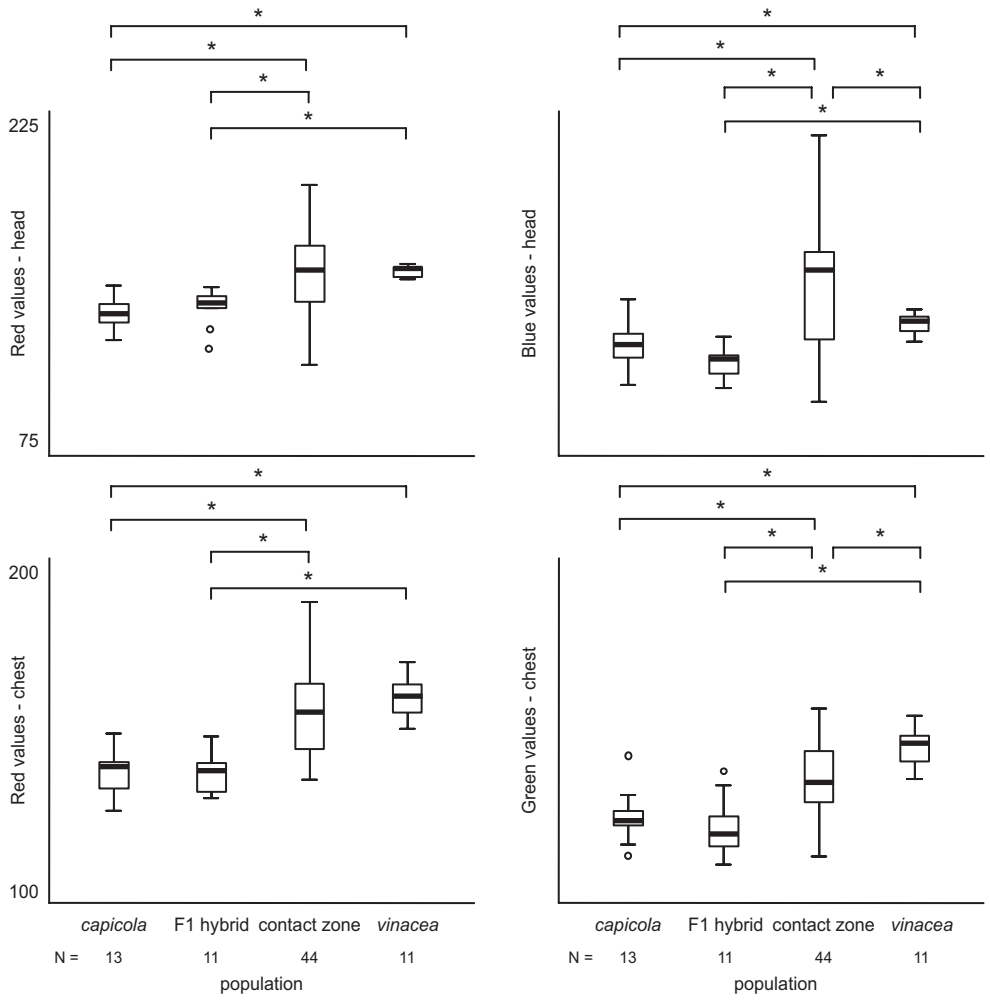


Figure 2.5 Four colour measures for *capicola*, F1 hybrids, contact zone and *vinacea* populations. Overall, *vinacea* and *capicola* are significantly different and F1 hybrids and contact zone individuals are intermediate. See Results for more details.

Vinacea had higher red, green and blue values than *capicola* for all measures. *Vinacea* also had higher values for all measures than F1 hybrids. *Capicola* did not differ in any measure from the F1 hybrid individuals. *Vinacea* did not differ in any value from the contact zone individuals except for the Head Blue and Chest Green values. *Capicola* had lower values than contact zone individuals in every measure. Contact zone individuals differed from F1 hybrids in every measure (Table 2.3 and Figure 2.5) and were more similar to *vinacea*. In summary, *vinacea* and *capicola* differed in their colour. Individuals from the contact zone were more similar to *vinacea* and different from *capicola* while F1 hybrids were more similar to *capicola* and different from *vinacea*.

Differences between the sexes within each group were also analyzed. For *vinacea* only one parameter showed a difference. Males (n=8) had higher values than females (n=3) in the Head Blue parameter (t_9=2.432, *P*=0.038). For *capicola*, using the field photos (11 males, 11

females) and the lab photos (5 males, 8 females) there were no significant differences. For the contact zone population (30 males, 14 females), males had higher values for Head Red (t_{42}=2.972, *P*=0.005), Head Green (t_{42}=3.612, *P*<0.001), Head Blue (t_{42}=3.992, *P*<0.001) and Chest Blue (t_{42}=2.771, *P*=0.008). In F1 hybrids (5 males, 6 females) there were no differences in colour measures between males and females. See Appendix, Table A2.5 for all tests and their *P* values.

Differences in colour of F1 hybrids depending on the composition of the parental individuals crossed to produce F1 hybrids were also analyzed. There were no differences between individuals from a cross of a *capicola* father and a *vinacea* mother (n=5) or a cross with a *vinacea* father and a *capicola* mother (n=6). See Appendix, Table A2.6 for all tests and their *P* values.

F1 viability

A total of 60 F1 individuals were bred in the lab; 34 females and 26 males, a sex ratio not different from 50/50 (χ^2=1.067, *P*=0.302). There were seven couples with a *capicola* father and a *vinacea* mother which bred 36 F1s; 24 females and 12 males. Nine couples consisting of a *vinacea* father and a *capicola* mother bred 24 F1s; 10 females and 14 males. Overall the percentage of fertilized eggs of the total eggs laid was 50.33%. Of the fertilized eggs on average 60.94% hatched. Of the hatched eggs on average 72.69% survived to adulthood. When looking at the two types of crosses, one with a *vinacea* father and a *capicola* mother, and one with a *capicola* mother and a *vinacea* father, there seem to be slight differences in fertility and hatchability. Cross type had a significant effect on the proportion of eggs fertilized ($F_{1,15}$=4.8872, *P*=0.0442). Confidence intervals (95%) for pairs with a *capicola* father are 0.6672-0.8347 and with a *vinacea* father are 0.4526-0.6240. Cross type did not have an effect on the proportion of eggs hatched ($F_{1,15}$=0.0058, *P*=0.9403) Confidence intervals (95%) for pairs with a *capicola* father are 0.4339-0.6563 and with a *vinacea* father are 0.4189-0.6510. Cross type had a significant effect on the proportion of young surviving to adulthood ($F_{1,15}$=5.5671, *P*=0.0334). Confidence intervals (95%) for pairs with a *capicola* father are 0.7315-0.9406 and with a *vinacea* father are 0.4886-0.789. Although these data cannot be treated as giving more than an indication, they suggest that, if anything, pairs with a *capicola* father and a *vinacea* mother have a somewhat higher fertility.

Discussion

Contact zone is a hybrid zone

Individuals from the contact zone were generally intermediate to the parental species in AFLP markers, morphological measures, and colour characteristics. Their vocal characteristics have already been shown to be intermediate (de Kort *et al.* 2002a). All in all, this qualifies them as hybrids. The contact zone population was genetically more similar to *vinacea* and consistently significantly different from *capicola*, but not from *vinacea*, in morphology and colour characters. F1 hybrids, which by definition are genetically truly intermediate, are different in colour and morphology from *vinacea* and not from *capicola*. Combined, this suggests that there is backcrossing

to *vinacea* in the hybrid population. The high frequency of hybrids with *capicola* mtDNA, an AFLP marker distribution that is more similar to *vinacea* than to *capicola*, and some *vinacea* individuals with *capicola* mtDNA haplotypes portray a hybrid zone with directional introgression.

Assignment and 'mixed' mitochondrial DNA

It was difficult to assign hybrids, both F1s and field hybrids, to different hybrid categories (F1, F2, or backcross). The simulations in AFLPOP showed that our markers were not diagnostic enough to discriminate between different hybrid categories. The likelihood results of our F1 individuals confirm this. Vähä and Primmer (2006) showed that with Bayesian classification methods it is difficult to detect F1 individuals and requires 12 or 24 loci with Fsts between hybridizing populations being 0.21 or 0.12 respectively, and even more loci to identify backcrosses. We expect this also roughly apply to our classifications based on likelihoods.

Three *vinacea* individuals and most individuals from the contact zone had *capicola* haplotypes. There were two individuals that had a mixed 'vcv' haplotype profile: one *vinacea* individual and one hybrid individual. The *vinacea* individual, V4, was sequenced at the COI gene and cyt b gene and seemed to have a 'mixed' haplotype. Further analyses are required to know whether these are cases of heteroplasmy, recombination or sequencing of nuclear genes. Mitochondrial pseudo genes and nuclear insertions (Numts) are common (Arctander 1995; Zhang & Hewitt 1996; Sorenson & Quinn 1998; Bensasson *et al.* 2001; Thalmann *et al.* 2004). This seems unlikely in this case because we aligned the sequences with known GenBank sequences to check for Numts. Also, all F1 individuals had the same haplotype 'profile' as their mothers, further verifying our results. Heteroplasmy could be occurring as it is detected more easily in areas of hybridization and has been detected in birds (Kvist *et al.* 2003). Extensive analyses are required to really understand the origin of the seemingly 'mixed' haplotypes.

Causes of hybridization

Hybridization demonstrates that possible premating barriers like vocalizations may be incomplete. *Vinacea* and *capicola* are sister species (Johnson *et al.* 2001) that are similar in appearance and this may have contributed to accepting the other species as potential partners. We have shown they differ slightly in plumage colour and morphological measures but these are slight compared to their signal differences. The most noticeable difference between *capicola* and *vinacea* males is their long range territorial vocalization, the perch coo (Slabbekoorn *et al.* 1999; de Kort *et al.* 2002a). They do not differ in their close range vocalization, the bow coo, which is used in the contexts of aggression and courtship (Slabbekoorn *et al.* 1999; de Kort *et al.* 2002a). Signal similarity has been shown to lead to hybridization (Qvarnström *et al.* 2006; Price 2008). Playback experiments have shown that males in allopatric populations of both species discriminate between conspecific, heterospecific and hybrid perch coos (de Kort *et al.* 2002b; Chapter 3). However, they do not discriminate between the bow coos (de Kort *et al.* 2002b). If this is also the case for females, then this vocalization may be just as effective in inter- as intraspecific interactions. It is unknown whether females first approach a male based on his territorial perch coo and then further base

their choice on the bow coo, or whether males approach females (at feeding or drinking sites) and start bow cooing. The latter explanation would make hybridization easier than the former, as females would not be exposed, at first, to the different species specific perch coo.

Despite vocal differences acting as potential premating barriers, a common cause of hybridization is difficulty in finding conspecific mates due to being the scarce species in an area of sympatry. This is known as Hubbs principle (Hubbs 1955). In birds females are often the choosy sex, and in an area of sympatry in which they cannot find conspecific mates, they may have to mate heterospecifically (Randler 2006) and in some cases this may even be adaptive (Veen *et al.* 2001). Due to the same selectivity, females of the common species, *vinacea*, might select against *capicola* males and favour conspecific males. Why males of the common species would mate with females of the rare species is less clear. It could be that they are less choosy, that they may outnumber conspecific females (Grant & Grant 1997b), or that they cannot discriminate between females of both species. As most individuals in the hybrid zone had *capicola* mtDNA, original matings that caused the hybridization must have involved a *capicola* female and a *vinacea* male. The hybrid zone is farther from the allopatric *capicola* population than from the allopatric *vinacea* population (270km vs 50km) and separated from it by unsuitable habitat, whereas it is directly connected to the *vinacea* population by continuous comparable habitat. So, it is very likely that hybridization started because *capicola* individuals dispersed into the *vinacea* population. If a few *capicola* individuals end up in the *vinacea* population (or at its edge) they are the rarer species. Hence, hybridization may have started with *capicola* females mating with *vinacea* males. Viability of the offspring may next have led to the establishment of a hybrid zone.

Hybrid fitness

A key issue in the stability of hybrid zones and consequently the maintenance or breakdown of species barriers is hybrid fitness (Rohwer *et al.* 2001). Endogenous selection against hybrids is usually expressed in reduced viability or fertility (Barton & Hewitt 1985; Rohwer *et al.* 2001). Although the F1 data are limited, they do not indicate that hybrids between *vinacea* and *capicola* are inviable. This is supported by the field data. The common occurrence of hybrids, but also the backcrosses to *vinacea* and introgression of *capicola* mtDNA suggest hybrids are fertile to a certain degree (Smith & Rohwer 2000) and selection may be favouring the spread of hybrid forms into the parental species range (Arnold 1997). Furthermore, in hybrids reduced fitness is usually expected in the heterozygous sex (females in birds, Haldane 1922; reviewed by Wu *et al.* 1996). Although this has been observed in various hybridizing birds (Tegelström & Gelter 1990; Sætre *et al.* 1999; Helbig *et al.* 2001), our lab F1 individuals showed no evidence of differences in viability between the sexes, as the sex ratio was not different from 50/50. Our findings are similar to those reported by Lijtmaer and colleagues (2003) for crosses between *S. decaocto* and *S. roseogrisea*. These sister species are in the same genus as our species pair and show a similar mtDNA divergence of 2.4% (Johnson *et al.* 2001; Lijtmaer *et al.* 2003). That study also reports that hatching success was similar in F1 hybrids and backcrosses, but that both F1 hybrids and backcrosses between more distant species resulted in decreased viability (see also Price & Bouvier 2002) and an increase in

the proportion of males due to female inviability in accordance with Haldane's rule (Lijtmaer *et al.* 2003). This is in line with what has been found elsewhere: in species with low divergence, like the ones studied here, there are no post zygotic barriers and hybrids are relatively fit (Smith & Rohwer 2000; Price & Bouvier 2002; Babik *et al.* 2003). Moreover, in birds, premating isolation mechanisms are expected to evolve before postmating isolation (Grant & Grant 1997a). In the absence of premating isolation, postmating isolation may not have evolved either and hybrids may be as fit as parental species individuals.

Exogenous selection against hybrids may result in hybrids having difficulty to defend a territory or finding a mate due to intermediate hybrid characters. Behavioural sterility due to dysfunctional hybrid signals has been found in insects (Coyne & Orr 2004; Gottsberger & Mayer 2007; Price 2008). Within the hybrid zone, hybrids may not be experiencing a loss of fitness. Hybrids may show a wide range of vocalizations including ones sounding like parental species (de Kort *et al.* 2002a and Chapter 5). Moreover, hybrid male territorial vocalizations are equally effective as parental vocalizations within the hybrid zone, indicating that the territorial vocalizations of hybrid males are suitable to successfully establish territories (Chapter 3). In the allopatric populations, parental species showed a weaker response to hybrid vocalizations compared to the response to species specific vocalizations (Chapter 3). However, hybrids sounding like one of the parental species, may not experience exogenous selection in both the hybrid zone and allopatric populations.

Asymmetric introgression

The high frequency of hybrids with *capicola* mtDNA, an AFLP marker distribution that is more similar to *vinacea* than to *capicola*, and some *vinacea* individuals with *capicola* mtDNA haplotypes indicate directional hybridization. Asymmetric introgression is found in many avian contact zones where it could indicate movement of the zone (Rohwer *et al.* 2001; Secondi *et al.* 2006) or one species being swamped by another (Pearson 2000; McDonald *et al.* 2001). In our zone the predominance of *capicola* mtDNA in the hybrid zone is most likely explained by the hybridization scenario discussed above, i.e. *capicola* as the rare species was more likely to mate heterospecifically. The proximity of the hybrid zone to the *vinacea* population, may also be the cause of the backcrossing with *vinacea,* as *vinacea* mates are more readily available (Grant 1993). However, there are two alternative scenario's that may also explain the asymmetrical introgression. The first is that Haldane's rule *is* at work here and female hybrids do experience a fitness loss, but only for hybrids resulting from one of the two interspecific crosses, namely a *vinacea* female with a *capicola* male. However our F1 data suggest, if anything, the opposite. This makes this scenario less likely, although differences in fecundity or mating behaviour may also exist between offspring of both reciprocal crosses, as has been found in butterflies (Davies *et al.* 1997) and grasshoppers (Virdee & Hewitt 1992). A second explanation is a behavioural mechanism concerning mate choice. *Capicola* females may prefer *vinacea* males or *vinacea* males may prefer *capicola* females more than the other way around. In *Streptopelia* doves, mate preferences are affected by sexual imprinting on the parental appearance (Brosset 1971; ten Cate *et al.* 1992), but may also show biases to preferring a

related species (ten Cate *et al.* 1992). We lack knowledge about mate choice for the current species, so cannot assess whether this may be at work here. Asymmetric introgression has also been associated with asymmetric behaviours in two hybridizing species, i.e. one of the species responds (aggressively) to the other, but not vice versa (Pearson & Rohwer 2000; McDonald *et al.* 2001). In other hybrid zones behavioural asymmetry matched introgression asymmetries (McDonald *et al.* 2001; Rohwer *et al.* 2001). In our two species territorial response of the two allopatric populations was shown to be symmetrical, both species respond with similar intensities to conspecific and heterospecific signals (de Kort *et al.* 2002b; Chapter 3). The territorial behaviour of the two parental species does not seem to explain the asymmetric introgression in this case.

Consequences of hybridization

We have established the presence of a clear, narrow, hybrid zone between *vinacea* and *capicola*. Hybrids are abundantly present in this zone, seem viable and there is evidence for backcrossing and introgression into *vinacea*, which is most likely due to a combination of geographical and behavioural factors. There is also directional introgression in this hybrid zone, most likely due to geographical location of the hybrid zone. Whereas in a lot of avian hybrid zones, there is some form of postmating isolation (Tegelström & Gelter 1990; Sætre *et al.* 1999; Helbig *et al.* 2001), there is no evidence for this in the *Streptopelia* hybrid zone, similar to other hybridizing species pairs (Grant & Grant 1998; Gee 2003). Directional introgression is also common to other species pairs (McDonald *et al.* 2001; Rohwer *et al.* 2001), especially if one of the hybridizing species is rare (Grant & Grant 1997b).

Speciation, the divergence of two taxa, is reversed by hybridization. However, complete reproductive isolation is the endpoint of the process of speciation (Bush 1993; Grant & Grant 1996). In birds, the potential to hybridize may remain 20 million years after divergence from a common ancestor (Prager & Wilson 1975) and many species are known to hybridize in nature (Grant & Grant 1992; Price 2008). Therefore, young species are expected to be capable of hybridizing when they come into contact. The younger the species, the lesser the fitness consequences of hybridization are (Price & Bouvier 2002). In birds, premating barriers are important in reproductive isolation and usually evolve before postmating barriers or act in the absence of postmating barriers (Grant & Grant 1997a; Price 2008). However, premating barriers only may not suffice, as even a low incidence of hybridization may cause species to slowly merge back into one, or 'despeciate' (Grant & Grant 2006; Price 2008). The fitness of hybrids may vary with ecological conditions and thereby change the direction of speciation (Grant & Grant 1998). These merge-diverge cycles (Grant & Grant 2006) may be more common in avian species. Development of postzygotic isolation seems an essential part of speciation and may only occur if species remain geographically isolated for a very long time (Price 2008). Hybrid zones such as the *Streptopelia* hybrid zone, may therefore not be a transient step on the path to complete speciation, but a step on the path to 'despeciate' or a permanent phase in which species remain distinct with a zone of hybridization between them. In the case of hybrid superiority, a new lineage may even arise.

Acknowledgements

We would like to thank the Uganda Wildlife Authority and the Uganda Council of Science and Technology for allowing us to export samples and live birds. We would like to express our gratitude to Staffan Bensch and Klaas Vrieling for coaching, assistance and advice on the molecular techniques. We would like to thank the following people for their assistance and/or support at some stage in the project: Martieneke and Robbert Faber, Marjolein and Guy Rijcken, Roelant Jonker, Jolijn Zegwaard, Henny Koolmoes, Peter Snelderwaard, Inke van der Sluijs, Christine Dranzoa, Sylvester Nyakaana, Andama Kennedy, Geoffrey, Kalyango Vincent, Ndaula Raymond and Eluku Nathan. Elles Lalieu helped with photographing the birds and measuring the colours. We would like to especially thank Harriet Nakabiito for all her help and advice in obtaining field assistance, housing doves before transport, helping with the paperwork and practical issues concerning the export of the doves from Uganda. PMdH was funded by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO 82-267). CtC acknowledges support by a Lorentz Fellowship from the Netherlands Institute for Advanced Study in the Humanities and Social Sciences (NIAS) during the preparation of the manuscript.

Appendix

Table A2.1 Sequences on three mitochondrial genes that were used to find restriction sites between *capicola* and *vinacea*. * Restriction site was determined after alignment with same genes in Chicken (see Methods).

	GenBank accession numbers							
mtDNA	*vinacea*	*capicola*	primers	sequences (5'-3')	size (bp)	restriction site between bp*	restriction enzyme	fragment size
Cyt b	AF353402	AF279709	Fcb1	TCTGCCTGCTAACCCAAATC	184	15086/15087	Bfa I	65/112
			Rcb2	CGTCCGATGTGCAGGTAGAT				
ND2	AF353423	AF353422	FNdeh1	ATCTAACGCCTGAGCTACCG	160	5572/5573	Rsa I	100/55
			RNdeh2	TAGTGCGGTGGTTATGGATG				
COI	AF353473	AF279734	Fcoxi2	CCTGGGCTTTATCGTTTGAG	237	7546/7547	HpyCH4 IV	47/186
			Rcoxi2	GAGTTTGCCAGGACAATTCC				

Table A2.2 Sample sizes, means and standard errors of morphology measures (bill length and tarsus length in mm) and colour measures (red, green and blue values measured on the head and on the chest) for each population.

	capicola - lab			*capicola* - field			F1 hybrid			contact zone			*vinacea*		
	N	mean	SEM	N	mean	SEM	N	mean	SEM	N	mean	SEM	N	mean	SEM
bill length (mm)	15	13.86	0.25				30	13.61	0.22	49	13.12	0.12	14	12.83	0.21
tarsus length (mm)	16	27.16	0.21				30	26.27	0.16	49	25.94	0.16	14	25.4	0.20
head red	13	136.10	2.15	22	130.62	2.33	11	138.76	2.65	44	154.60	2.58	11	156.06	0.81
head green	13	126.43	2.47	22	135.19	3.03	11	121.72	1.85	44	143.18	2.87	11	139.28	0.94
head blue	13	119.59	3.18	22	147.00	3.93	11	111.35	2.24	44	149.66	4.42	11	130.43	1.51
chest red	13	136.92	1.95	22	142.57	2.41	11	136.03	2.00	44	156.09	2.05	11	160.57	2.03
chest green	13	122.41	2.23	22	122.10	2.13	11	119.12	2.79	44	134.28	1.85	11	144.37	1.98
chest blue	13	112.58	2.58	22	112.52	2.49	11	110.13	3.20	44	133.75	2.41	11	135.55	3.01

Table A2.3 Sex differences in morphology measures for all populations.

population	parameter	sex	n	mean (mm)	SEM	t	df	*P*
Vinacea	bill length	male	8	12.75	0.30	-0.43	12	0.675
		female	6	12.94	0.32			
	tarsus length	male	9	25.74	0.67	2.95	12	0.012
		female	5	24.77	0.38			
capicola	bill length	male	7	13.57	0.41	-1.093	13	0.294
		female	8	14.11	0.29			
	tarsus length	male	8	27.68	0.24	3.201	14	0.006
		female	8	23.64	0.22			
contact zone	bill length	male	32	13.14	0.88	0.247	47	0.806
		female	17	13.08	0.83			
	tarsus length	male	32	26.2	1.14	2.288	47	0.027
		female	17	25.44	1.00			
F1 hybrid	bill length	male	15	12.99	0.22	-3.362	28	0.002
		female	15	14.24	0.30			
	tarsus length	male	15	26.33	0.21	0.365	28	0.718
		female	15	26.21	0.25			

Table A2.4 Differences in parental composition for F1 morphology measures.

population	parameter	father	n	mean (mm)	SEM	t	df	*P*
F1 hybrid	bill length	*capicola*	15	13.48	0.25	-0.608	28	0.548
		vinacea	15	13.75	0.36			
	tarsus length	*capicola*	15	25.99	0.23	-1.747	28	0.092
		vinacea	15	26.54	0.21			

Table A2.5 (opposite page). Sex differences in colour measures (red, green, blue values) for all populations. An * indicates groups that have unequal variances.

population	parameter	sex	n	mean	SEM	t	df	*P*
vinacea	head red	male	8	156.60	0.95	1.11	9	0.297
		female	3	154.60	1.52			
	head green	male	8	139.53	1.23	0.42	9	0.686
		female	3	138.60	1.36			
	head blue	male	8	132.28	1.32	2.43	9	0.038
		female	3	125.52	3.00			
	chest red*	male	8	160.71	2.81	0.16	8.99	0.878
		female	3	160.21	1.43			
	chest green*	male	8	143.41	2.67	-1.26	8.22	0.244
		female	3	146.94	0.86			
	chest blue	male	8	136.25	4.01	0.37	9	0.723
		female	3	133.67	3.70			
*capicola*_lab	head red	male	5	137.15	4.96	0.37	11	0.715
		female	8	135.43	1.99			
	head green	male	5	129.16	5.51	0.87	11	0.405
		female	8	124.73	2.24			
	head blue	male	5	125.00	5.87	1.40	11	0.190
		female	8	116.21	3.41			
	chest red	male	5	139.06	3.06	0.86	11	0.410
		female	8	135.58	2.58			
	chest green	male	5	124.98	5.09	0.90	11	0.386
		female	8	120.81	1.90			
	chest blue	male	5	117.00	5.24	1.41	11	0.187
		female	8	109.82	2.44			
*capicola*_field	head red	male	11	129.72	3.30	-0.38	20	0.707
		female	11	131.53	3.43			
	head green	male	11	138.27	3.97	1.02	20	0.321
		female	11	132.12	4.57			
	head blue	male	11	152.29	4.89	1.37	20	0.185
		female	11	141.71	5.95			
	chest red	male	11	146.28	3.75	1.60	20	0.126
		female	11	138.86	2.74			
	chest green	male	11	122.91	2.54	0.38	20	0.711
		female	11	121.28	3.54			
	chest blue	male	11	112.64	3.20	0.05	20	0.960
		female	11	112.39	3.98			
contact zone	head red	male	30	159.41	2.90	2.97	42	0.005
		female	14	144.29	4.11			
	head green	male	30	149.43	3.01	3.61	42	0.001
		female	14	129.79	4.66			
	head blue	male	30	160.04	4.81	3.99	42	<0.001
		female	14	127.41	6.05			
	chest red	male	30	156.18	2.33	0.06	42	0.949
		female	14	155.89	4.21			
	chest green	male	30	135.32	2.07	0.82	42	0.416
		female	14	132.05	3.78			
	chest blue	male	30	138.00	2.63	2.77	42	0.008
		female	14	124.66	4.24			
F1 hybrid	head red*	male	5	134.13	4.90	-1.63	5.047321	0.164
		female	6	142.62	1.77			
	head green	male	5	120.63	3.83	-0.52	9	0.619
		female	6	122.63	1.56			
	head blue*	male	5	111.80	4.82	0.16	5.003589	0.880
		female	6	110.98	1.71			
	chest red	male	5	135.13	3.16	-0.39	9	0.704
		female	6	136.78	2.79			
	chest green	male	5	120.47	5.02	0.42	9	0.682
		female	6	117.99	3.34			
	chest blue	male	5	110.93	5.53	0.22	9	0.832
		female	6	109.45	4.12			

Table A2.6 Differences in parental composition for F1 colour measures (red, green and blue values). An * indicates groups that have unequal variances.

population	parameter	father	n	mean	SEM	t	df	*P*
F1 hybrid	head red	*capicola*	5	139.18	3.39	0.14	9	0.892
		vinacea	6	138.40	4.25			
	head green	*capicola*	5	121.29	2.20	-0.20	9	0.845
		vinacea	6	122.08	3.05			
	head blue	*capicola*	5	109.64	2.36	-0.68	9	0.513
		vinacea	6	112.79	3.72			
	chest red*	*capicola*	5	134.84	1.90	-0.55	7.615598	0.595
		vinacea	6	137.02	3.45			
	chest green*	*capicola*	5	117.27	2.22	-0.63	6.894693	0.550
		vinacea	6	120.66	4.91			
	chest blue*	*capicola*	5	108.37	2.93	-0.51	7.436103	0.623
		vinacea	6	111.59	5.56			