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ORIGINAL PAPER

Occurrence and extent of hybridisation between the invasive Mallard Duck and native Yellow-billed Duck in South Africa

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Abstract Hybridisation between invasive and native species represents a significant threat to biodiversity. The Mallard Duck (*Anas platyrhynchos*) is known to hybridise with numerous closely related *Anas* species in regions where they have been introduced, threatening the genetic integrity of native ducks and in some instances contributing to their extinction risk. Mallard Ducks were introduced into South Africa in the 1940s and are now naturalised and widespread in the country. It has been speculated that Mallard Ducks are hybridising with native Yellow-billed Ducks (*A. undulata*) in South Africa, but evidence for this remains observational or purely anecdotal. Here we use data from nuclear microsatellite markers and mitochondrial DNA sequencing to show that hybridisation is indeed occuring between these two species. We found evidence for the occurance of hybridisation, mostly as crosses between Mallard Duck hens and Yellow-billed Duck drakes. Surprisingly, our results suggest that introgressive hybridisation is primarily occuring into the invasive Mallard Duck population (mostly Mallard Duck backcrosses were detected), evidenced by directionally-skewed gene flow and sex-biased mating. Whether these findings reflect true assortative mating or a case of Haldane's rule remains unknown. We also found evidence of high connectivity between Yellow-billed Duck populations, as far as 1000 km apart, in South Africa. Taken together these results suggest that hybrid genotypes can disperse over vast distances between populations and lead to genetic pollution, even in the absence of invasive Mallard Ducks. Active management of Mallard Duck populations has been met by public resistance in some areas in South Africa, partly because of a lack of evidence showing clear impacts by these birds. This study provides some of the first scientifically-documented evidence for such impacts.

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Introduction

Invasive species are a significant driver of global change and pose a serious threat to biodiversity (Clavero and Garccıa-Berthou 2005; McGeoch et al. 2010; Blackburn et al. 2019). Invader impacts often manifest through the displacement of natives via ecological processes like competition or predation (Blackburn et al. 2014). However, evolutionary impacts, such as introgressive hybridisation, are also recognised as a major impact on native populations (Rhymer and Simberloff 1996; Blackburn et al. 2014). Indeed, hybridisation is ranked as one of the top ten impacts by invasive species, often leading to native population declines and even extinction (Rhymer and Simberloff 1996; Wolf et al. 2001; Blackburn et al. 2014; Todesco et al. 2016; Kumschick et al. 2017). Hybridisation between closely related species often results in genetic introgression (Rhymer and Simberloff 1996; Schulte et al. 2012), therefore diluting native species' genepools and purging their populations of locally adapted genotypes (Allendorf et al. 2001; Olden et al. 2004; Schulte et al. 2012), and ultimately paving the road to extinction (Rhymer and Simberloff 1996; Wolf et al. 2001; Todesco et al. 2016).

Hybridisation between native and invasive species is usually managed through the removal of putative hybrids and the invasive species (Rieseberg 1991; Hughes et al. 2006; Robertson et al. 2015). However, hybrid individuals are not always easy to identify based on morphology alone and thus genetic monitoring is often used to detect hybridisation, identify hybrids and determine whether introgression is occurring (Schwartz et al. 2006; Vähä and Primmer 2006; Devillard et al. 2014; van de Crommenacker et al. 2015). Moreover, the extent of hybridisation and introgression is likely to be underestimated based on observational data alone, as it is very difficult to determine levels of hybridisation (e.g. F1 vs F2 hybrids) and backcrossing based on morphology alone (Allendorf et al. 2001; Vähä and Primmer 2006).

A poster child for evolutionary impacts on natives through hybridisation with invasive species is the Mallard Duck, *Anas platyrhynchos* Linnaeus, 1758. First introduced as hunting targets in the eastern United States of America and New Zealand, and then as a popular ornamental species across the world (Long 1981; Rhymer 2006); Mallard Ducks have become established and invasive, in three countries (Australia, New Zealand and South Africa) and throughout the Hawaiian Islands (Rhymer et al. 1994; Fowler et al. 2009; Guay and Tracey 2009; Government of the Republic of South Africa 2016). Invasive Mallard Ducks readily hybridise with several closely related natives in the genus *Anas*; including the Florida Mottled Duck (*A. fulvigula*), the American Black Duck (*A. rubripes*), the New Zealand Grey Duck (*A. superciliosa superciliosa*) and the Hawaiian Duck (*A. wyvilliana*) (Rhymer et al. 1994; Kulikova et al. 2004; Mank et al. 2004; Fowler et al. 2009). Many of these hybrids are fertile with evidence of genetic introgression (e.g. in the American Black Duck, Mank et al. 2004; New Zealand Grey Duck, Rhymer et al. 1994). Hybridisation between Mallard Ducks and native duck species is often sex-biased. In some instances, aggressive Mallard Duck males have been found to outcompete native drakes for available native hens (Brodsky and Weatherhead 1984; Seymour 1990; D'Eon et al. 1994). In other instances, mating is biased towards Mallard Duck hens and native drakes, as is the case for Hawaiian Ducks (Fowler et al. 2009).

Observational data suggest that Mallard Ducks are able to hybridise with numerous native South African *Anas* species, notably Cape Shovelers (*A. smithii*), Cape Teal (*A. capensis*), African Black Ducks (*A. sparsa*), and especially, Yellow-billed Ducks (*A. undulata*) (Dean 2000; Owen et al. 2006; Stafford 2010). Mallard Ducks were introduced into South Africa in the 1940s and naturalised populations quickly became widespread, likely representing the early stages of a large scale invasion (Liversidge 1985; Stafford 2010; South African Bird Atlas Project 2 2019a). Mallard Ducks are mainly found in the southern parts of the Western Cape Province and in the North Eastern parts in the Gauteng Province, but have been recorded in all other seven provinces of the country (Fig. 1; Stafford 2010; South African Bird Atlas Project 2 2019a). Mallard Ducks are currently listed as a category two invasive species under South

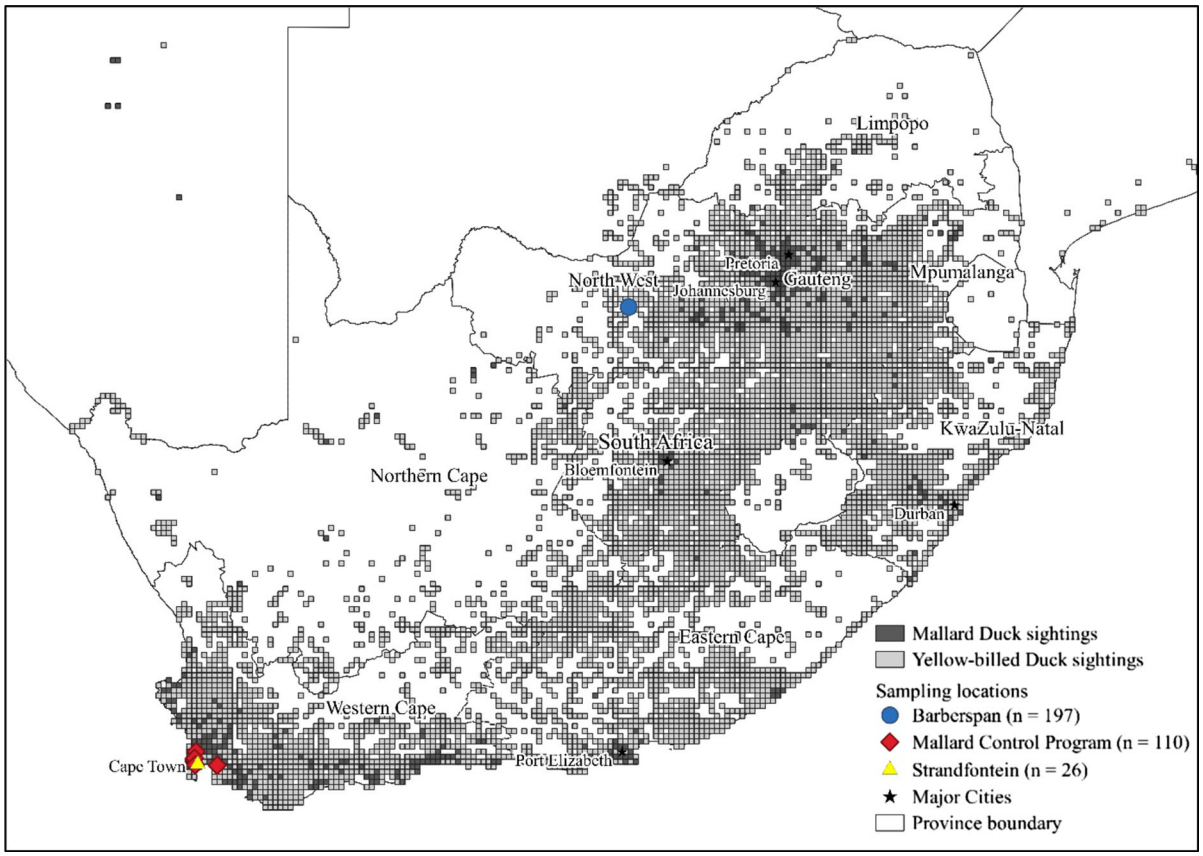

Fig. 1 Occurrence records of Mallard Ducks and Yellow-billed Ducks in South Africa (data collected between 2007 and 2019; South African Bird Atlas Project 2 2019a, b) and sampling locations included in this study

Africa's National Environmental Management Biodiversity Act (Act 10 of 2004: Alien and Invasive Species Regulations), meaning that a permit is required to keep them and that they must be controlled in areas not falling under permits (Government of the Republic of South Africa 2014). The native Yellow-billed Duck, *Anas undulata* Dubois, 1839, is a widespread species in south-eastern Africa (Maclean 1997; South African Bird Atlas Project 2 2019b) and is a common resident in South Africa with no regular migrations (Brown et al. 1983; Oatley and Prys-Jones 1986; Maclean 1997). Although Yellow-billed Ducks are not threatened per se, the potential for hybridisation with invasive Mallard Ducks is becoming a concern (Dean 2000; Owen et al. 2006; Stafford 2010). Observational accounts of hybrids between the two species have been reported in the Eastern Cape, Western Cape, Free State, KwaZulu-Natal and Gauteng Provinces (Brooke 1989; Dean 2000). Yellow-billed Ducks are likely to be attracted to Mallard Ducks due to the showiness of Mallard Duck drakes and the similar calls and resemblance of hens of the two species (Skead 1980; Brooke 1986; Joubert 2009). If these two duck species are able to produce fertile offspring, then Mallard Ducks potentially pose a significant threat to the long-term genetic integrity of Yellow-billed Ducks in South Africa (Gray 1958; Johnsgard 1960; Stafford 2010). In addition, Mallard Ducks also compete with Yellow-billed Ducks for available habitat and food, often dominating ponds and displacing Yellow-billed Ducks (Banks et al. 2008). This, however, is unlikely to pose an immediate threat to the very large resident Yellow-Billed Duck population in South Africa, but may change in the future.

Given the perceived genetic impacts of Mallard Ducks on native Yellow-billed Ducks, and in accordance with South African law, a control program was

initiated in Cape Town in 2011 (Davies et al. 2020). However, this program has been met by strong public opposition (Banks et al. 2008; Stafford 2010) as many residents enjoy feeding Mallard Ducks and even consider free ranging individuals as 'pets'. Control methods are also perceived by the public as inhumane and cruel, with some residents having threatened officials trying to remove Mallard Ducks. A general lack of awareness of the potential threat that Mallard Ducks pose to native biodiversity may contribute to the negative sentiment that the general public hold towards the control program (Stafford 2010). Such opposition by the public makes it challenging to run an effective control program. Additionally, the current lack of convincing scientific evidence of the impacts of Mallard Ducks on native species further contributes to the inability of authorities in Cape Town to gain public support for Mallard Duck control programs. Strong public opposition has previously led to the failure of invasive species eradication programs. For example, the eradication of grey squirrels (*Sciurus carolinensis*) was rendered unsuccessful due to public opposition in Italy (Bertolino and Genovesi 2003). This particular program received strong opposition from animal rights activists (McNeely 2001), with the matter ending up in court and delaying eradication efforts by three years. This delay provided grey squirrel populations with enough time to reach densities and distributions that made eradication no longer feasible (Bertolino and Genovesi 2003).

This study aims to provide the first empirical assessment of the occurrence and extent of hybridisation between Mallard Ducks and native Yellow-billed Ducks in South Africa. We used nuclear microsatellite DNA markers to determine the genetic diversity of three widespread Yellow-billed Duck populations in South Africa and whether they represent one or multiple structured populations. We hypothesised that populations of Yellow-billed Ducks will show low levels of inbreeding and low population genetic structure, as they are widespread and migration could occur between populations. Secondly, to assess whether hybridisation between Mallard and Yellow-billed Ducks is occurring, we used nuclear microsatellite DNA markers and mitochondrial DNA sequencing data to identify putative hybrid individuals and determine whether introgression is occurring and, if so, whether sex-biased mating is prevalent between Mallard and Yellow-billed Ducks. We hypothesised that there is extensive hybridisation and introgression between Mallard and Yellow-billed Ducks with more introgression occurring into the Yellow-billed Duck population. We also hypothesised that sex-biased mating is occurring, with a higher incidence of mating between Mallard Duck drakes and Yellow-billed Duck hens than between Mallard Duck hens and Yellow-billed Duck drakes.

Materials and methods

Sampling and DNA extraction

Sampling for this study was approved by Stellenbosch University's Research Ethics Committee (Application number: SU-ACUD17-00042). Blood and feather samples of Mallard Ducks and putative Mallard Duck × Yellow-billed Duck hybrids were obtained from a Mallard Duck control program conducted by the City of Cape Town between 2015 and 2018 (n = 121; Fig. 1). Ducks were sedated using alpha-chloralose placed in bread and were then moved to a suitable onsite location where blood samples were taken and euthanasia conducted by a qualified veterinarian. For each individual bird, at least four flight feathers were removed for additional DNA material and a photograph taken for classification (i.e. Mallard Duck or putative hybrid). Morphological features used to identify putative hybrids included intermediate plumage, yellow feet pigmentation, green head plumage and curled tail feathers. Blood and feather samples were stored at − 80 °C until further use.

Yellow-billed Duck blood (stored in Queen's buffer) and feather samples (n_{total} = 234) were collected in 2013 and 2014. Two-hundred and two samples were collected from Barberspan in the North West Province (− 26.586713S, 25.581849E; Fig. 1) and 32 samples were from Strandfontein (False Bay Nature Reserve; − 34.072234S, 18.516217E; Fig. 1) in the Western Cape Province. These birds were captured using standard mistnetting and walk-in trapping procedures. Individuals were released without harm after blood and feathers were collected from these birds. Individuals from the Mallard control program that were determined to be Yellow-billed Ducks using genetic analyses (see "Results" section), were also included in the Yellow-billed Duck dataset [henceforth referred to as the Marina Da Gama

population (− 34.085389S, 18.469881E)] while those classified as hybrids were removed.

For all samples, DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, supplied by Whitehead Scientific, Cape Town, South Africa) following the manufacturer's protocol with slight modifications. For the blood samples, up to 100 µl of blood was used, and the incubation time for proteinase K digestion was extended to 1 h with vortexing every 15 min. For the feather samples, a 2–5 cm piece was cut from the base of larger feathers and for smaller feathers the entire calamus was included. The base of the feather was frozen using liquid nitrogen before being cut up into small pieces and crushed. We added 20 µl 1 M DTT solution, 20 µl proteinase K and 300 µl Buffer ATL to crushed feather material, before overnight incubation at 56 °C. Seven feather samples with low yielding DNA concentrations using the protocol described above were extracted using a DNA salt extraction protocol (MacManes 2013). DNA quantity and quality for all extractions was determined using a nanodrop (Nanodrop, Thermo Fisher Scientific, Waltham, Massachusetts, United States) and samples were frozen at − 80 °C until further use. In total we extracted DNA from 355 ducks.

Microsatellite genotyping and data preparation

Twenty-eight microsatellites, previously developed for Mallard Ducks and other *Anas* species, were selected for initial PCR optimization (Paulus and Tiedemann 2003; Denk et al. 2004; Huang et al. 2005; Hsiao et al. 2008). Twelve primer pairs that consistently gave good amplification in both Mallard and Yellow-billed Ducks were selected, and the forward primer of each labelled fluorescently based on size and annealing temperature. This allowed us to divide primers into two multiplexes containing six loci each (Table S1). Each multiplex was amplified in a 15 µl PCR reaction containing 1.5 µl of DNA (2 ng.µl^{-1}), 1.5 µl of primer mix (concentration of each primer provided in Table S1), 7.5 µl KAPA2G Fast Multiplex Mix (Kapa Biosystems, Cape Town, South Africa) and 4.5 µl PCR-grade H_2O. PCR conditions were: an initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, elongation at 72 °C for 25 s; a final elongation at 72 °C for 1 min. We included repeats for ca. 13% of all samples to check repeatability of microsatellite genotyping. Purified PCR fragments were separated using an ABI PRISM 3100 Genetic analyser (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and sized using the GENE-SCAN-500 (-250) as an internal size standard (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Allele sizes were scored using GeneMarker (Version 2.6.7; SoftGenetics LLC, Pennsylvania, USA). The final microsatellite data set was checked for null alleles using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). Null alleles were detected and consequently the software FreeNA (Chapuis and Estoup 2007) was used to calculate the frequency of null alleles at each locus and corrected (i.e., without null alleles using the ENA method described in Chapuis and Estoup (2007)) and uncorrected estimates of pairwise F_{ST} values (Weir 1996).

Samples that had 50% or more missing data were removed from all further downstream analyses, resulting in a data set consisting of 333 samples (Barberspan (n = 197), Strandfontein (n = 26) and Mallard Control Program (n = 110) (Table S2). The Yellow-billed Duck data consisted of three populations (Barberspan: n = 191; Strandfontein: n = 24; Marina Da Gama: n = 5).

Yellow-billed Duck genetic diversity and structure

Allelic richness (A_R), inbreeding coefficient (F_{IS}), and observed and expected heterozygosity (H_O and H_E) were calculated for each Yellow-billed Duck population using the diveRsity R package (version 1.9.90, Keenan et al. 2013; R Development Core Team 2017). Rarefaction was applied for A_R calculations due to the large differences in population sizes. Private allelic richness (PA_R) was calculated per population with rarefaction using the program ADZE (Allelic Diversity Analyzer; version 1.0; Szpiech et al. 2008). We also tested for allele frequency departures from Hardy–Weinberg equilibrium (HWE) expectations using the package pegas (version 0.11, Paradis 2010) in R (R Development Core Team 2017). Population pairwise F_{ST} values were calculated using GenAlEx (version 6.5, Peakall and Smouse 2012). To assess the distribution of genetic variation between and among Yellow-billed Duck populations, an analysis of molecular variance (AMOVA) with 999 permutations was performed using GenAlEx (version 6.5, Peakall and Smouse 2012).

We used STRUCTURE version 2.3.4 (Pritchard et al. 2000) to determine the number of genetic clusters (K) present in Yellow-billed Ducks. Samples classified as hybrids by NEWHYBRIDS (see "Results" section below) were removed prior to the analysis. One locus (APT001) was removed from this data analysis as it was monomorphic across all Yellow-billed Duck samples. We tested values of K between 1 and 5 (number of populations, plus 2). We used an admixture model with correlated allele frequencies, 100,000 burn-in iterations, 1,000,000 Markov Chain Monte Carlo repetitions and 20 independent runs for each K value. STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012) was used to determine to optimum number of K using the delta K method of Evanno et al. (2005). CLUMPP version 1.1.2 (Jakobssen and Rosenberg 2007) was used to process the 20 independent runs for each K value. DISTRUCT version 1.1 (Rosenberg 2004) and a Principal Components Analysis (PCA) using the R packages ade4 (version 1.7-11, Thioulouse et al. 1997) and adegraphics (version 1.0-10, Siberchicot et al. 2017) were then used to visualise population genetic structure. We also estimated the rate of gene flow between Yellow-billed Duck populations in Barberspan and Cape Town (Strandfontein and Marina Da Gama) using BayesAss (version 3.0.4, Wilson and Rannala 2003). For this analysis we used the mixing parameters dM = 0.05, dA = 0.2, dF = 0.2. The number of MCMC iterations was 1,000,000 with a burn-in of 10,000 and a sampling interval of 1000.

Assessing the occurrence and extent of hybridisation between Yellow-billed and Mallard Ducks

We used the full dataset in a second STRUCTURE analysis to identify putative hybrid individuals. Assignment values (q_{ik}) in STRUCTURE are calculated as the proportion of an individual's genotype that belongs to each of the identified optimal genetic clusters. We ran STRUCTURE to test values of K from 1 to 7 (number of sampling locations, plus 2), using the same parameters as described above for the Yellow-billed duck analysis.

Assignment values (q_{ik}) retrieved in STRUCTURE are dependent on the validity of the assumed model priors and the loci used. Consequently, to confirm the identification of hybrid individuals and to assign every individual to a specific genotype class (i.e. pure Mallard Duck, pure Yellow-billed Duck, F1, F2, Mallard Duck backcross, and Yellow-billed Duck backcross), we used the program NEWHYBRIDS (version 1.1 beta; Anderson and Thompson 2002). NEWHYBRIDS is a model-based Bayesian clustering method that uses multi-locus data to identity hybrid individuals and does not require reference samples of parental individuals (Anderson and Thompson 2002). We considered all six genotype classes in the initial analysis. No prior information regarding the class of individuals was used. We ran the analysis using both Jeffery's prior and the Uniform prior and for both theta (allele frequencies) and pi (mixing proportion), but we only report on Jeffery's Prior here as the results based on this prior were supported by our simulation analysis (see "Results" section below). We used a burn-in of 30,000 MCMC sweeps, followed by 50,000 MCMC sweeps for all models. Individuals were assigned to a genotype class if the posterior probability was greater than or equal to 0.8. If no genotype class with posterior probability of 0.8 or greater was identified then the individual was considered a hybrid of mixed ancestry. A second analysis was conducted excluding the F2 genotype class and a third analysis including a third generation of hybrid crosses. The genotype classes from the analysis that excluded the F2 genotype class were used for all further analyses because this analysis was able to differentiate backcrossed individuals.

No F1 individuals were identified by NEWHYBRIDS (see "Results" section below), consequently, to validate the genotype classes assigned by NEWHYBRIDS, we created and analysed a simulated dataset to determine 95% confidence intervals for assignment values (q_{ik}) for each genotype class. We selected 36 individuals for each parental population representing pure Mallard Ducks and pure Yellow-billed Ducks that had a greater than or equal to a 0.99 STRUCTURE assignment value (q_{ik}) to the Mallard or Yellow-billed Duck genetic cluster (see "Results" section). From these data a genotype dataset of 100 individuals was simulated for each genotype class (F1 hybrids, F2 hybrids, F1 hybrids backcrossed with Mallard Ducks and F1 hybrids backcrossed with Yellow-billed Ducks) using the function hybridise in the R package adegenet (version 2.1.1, Thibaut Jombart 2008; R Development Core Team 2017). Simulated genotypes were analysed in STRUCTURE using the same settings as for the full dataset but

limiting K to two clusters and 95% confidence intervals around q_{ik} values for assignment to the Mallard Duck genetic cluster were calculated for each simulated genotype.

Assessing directionality of Yellow-billed/Mallard Duck introgression and sex-biased mating

We estimated the rate of gene flow between Mallard and Yellow-billed Ducks using BayesAss (version 3.0.4, Wilson and Rannala 2003). We used the mixing parameters dM = 0.05, dA = 0.15, dF = 0.15. The number of MCMC iterations was 1,000,000 with a burn-in of 10,000 and a sampling interval of 1000. We defined the Mallard Duck group as individuals classified by NEWHYBRIDS as Mallard Duck or Mallard Duck backcross and the Yellow-billed Duck group as individuals classified as Yellow-billed Duck or Yellow-billed Duck backcross.

We sequenced part of the mitochondrial ND2 gene region to determine whether sex-biased mating is occurring between Mallard and Yellow-billed Ducks, by inferring whether mitochondrial haplotypes in putative hybrid and backcrossed individuals were of Mallard or Yellow-billed Duck origin. PCR amplification was done using the primers L5216 and H5766 (Sorenson et al. 1999). Each reaction 30 µl volume contained 3 µl of DNA (10 ng μl^{-1}), 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM dNTPs (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 0.2 mg ml^{-1} BSA (Promega, Madison, Wisconsin, United States), 3 µl of 10 × PCR reaction buffer and 3 U of *Taq* polymerase (Super-Therm JMR-801; Hoffman-La Roche, Basel, Switzerland). PCR conditions followed an initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, elongation at 72 °C for 1 min; and a final elongation at 72 °C for 10 min. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced using the forward primer only. We sequenced the mtDNA of three pure Mallard and three pure Yellow-billed Duck individuals, i.e. individuals with a probability value greater than 0.99 of being assigned to the each class according to the NEWHYBRIDS analysis (see above). In addition, 32 hybrid individuals were sequenced of which 14 were assigned to a specific hybrid genotype class by NEWHYRIDS (i.e. backcrossed Mallard Duck, backcrossed Yellow-billed Duck or F2). Sequences were edited and aligned in BioEdit (version 7.2.5, Hall 1999). The "pure" individuals were used to determine whether mitochondrial haplotypes in hybrid and backcrossed individuals were of Mallard Duck or Yellow-billed Duck origin. PopART (version 1.7.2, Leigh and Bryant 2015) was used to visualise the sequences in a haplotype network.

Results

Yellow-billed Duck genetic diversity and structure

Microsatellite genotype repeatability was 100% across all 46 replicated genotypes. Levels of observed and expected heterozygosity were similar in Yellow-billed Ducks (H_O: 0.39–0.45; H_E: 0.45–0.50; Table 1) and therefore levels of inbreeding low (F_{IS}: − 0.04 to 0.19; Table 1). On the other hand, allelic richness and private allelic richness measures were low (A_R: 2.92–3.27; PA_R; 0.40–0.51; Table 1). Three loci (APT004, APT014 and CAUD004) had allele frequencies deviating from those expected under HWE expectations for at least three populations. We found no significant difference between null allele-corrected and uncorrected pairwise F_{ST} values (Wilcoxon signed-rank test: Z = 382, $p = 0.280$) and a low mean null allele frequency ($\bar{x} = 0.076$, standard deviation = 0.087). All further analyses were therefore conducted using uncorrected data. The pairwise F_{ST} value between Barberspan and Strandfontein was significant, however all pairwise F_{ST} values were low ($F_{ST} < 0.02$; Table 2) and the AMOVA results showed that the majority of genetic diversity resided within individuals (68%) followed by among individuals (31%) and that only 1% resided between populations ($p = 0.001$).

Three genetic clusters ($K = 3$) were inferred as the optimal number of clusters for Yellow-billed Ducks (Figure S1). However, the STRUCTURE plot showed that all individuals contained assignment to all clusters, implying weak differentiation (Figure S2). This indicates that there is likely to be no observable genetic structure between the populations that we tested (also see Le Roux et al. 2008), a notion supported by the PCA plot (Figure S2).

The inferred estimate of gene flow from Barberspan to Cape Town is 0.3056 Yellow-billed migrants per generation (95% credible interval 0.2650–0.3462),

Table 1 Population genetic diversity parameters (mean across loci) for the three Yellow-billed Duck populations included in this study: number of samples (N); allelic richness (A_R); observed (H_O) and expected heterozygosity (H_E); inbreeding coefficients (F_{IS}); and private allelic richness (PA_R)

Population	N	A_R	H_O	H_E	F_{IS}	PA_R
Barberspan	191	2.92 (0.99)	0.41 (0.24)	0.50 (0.25)	0.19 (0.27)	0.40 (0.22)
Strandfontein	24	2.94 (1.09)	0.39 (0.28)	0.49 (0.25)	0.19 (0.33)	0.41 (0.28)
Marina Da Gama	5	3.27 (1.19)	0.45 (0.20)	0.45 (0.21)	− 0.04 (0.24)	0.51 (0.33)

Standard deviations are indicated in brackets

Table 2 Pairwise F_{ST} values calculated using an AMOVA between various Yellow-billed Duck groups

Population	Barberspan	Strandfontein
Strandfontein	0.010* (0.009)	
Marina Da Gama	0.014 (0.154)	0.011 (0.213)

p values are included in brackets and significant values ($p < 0.05$) are indicated with an *

while the estimate for gene flow from Cape Town to Barberspan is much lower at 0.0072 migrants per generation (95% credible interval − 0.0020 to 0.0164).

Assessing the occurrence and extent of hybridisation between Yellow-billed and Mallard Ducks

For the entire dataset, we identified two genetic clusters, corresponding to Mallard and Yellow-billed Duck clusters (Figure S3). Pure Mallard Ducks (assignment value (q_{ik}) greater than or equal to 0.99) contained 27 private alleles whereas Pure Yellow-billed Ducks had 32 private alleles. The STRUCTURE analysis identified 35 putative admixed individuals (assignment value (q_{ik}) to either Mallard or Yellow-billed Duck genetic cluster < 0.9). Twenty nine of these individuals originated from the ongoing Mallard Duck control program in Cape Town. Six admixed individuals were also identified in the Yellow-billed Duck populations sampled from Strandfontein and Barberspan. The different levels of admixture provides evidence that hybridisation and backcrossing have possibly been occurring in South Africa for several generations (Fig. 2a,b).

Assignment tests in STRUCTURE and NEWHYBRIDS were largely congruent (see Figure S4). In total, our NEWHYBRID analysis identified 67 putative hybrid individuals (analysis with the genotype class F2 excluded; Fig. 3, Table S3). Of these, none were F1 individuals, 20 had a posterior probability > 0.8 of being backcrossed to either Mallard or Yellow-billed Ducks, 15 were classified as Mallard Duck backcrosses, and five as Yellow-billed Duck backcrosses. The remaining 47 individuals could not be assigned to a genotype class with high levels of certainty and were therefore classified as 'hybrids of mixed ancestry'.

Fifty-four percent of individuals obtained from the Mallard Duck control program in Cape Town represented hybrids based on the NEWHYBRIDS assignments. Five individuals that were initially classified as hybrids based on morphology (yellow feet pigment) are most likely pure Yellow-billed Ducks according to NEWHYBRIDS (mean posterior probability of being assigned to the Yellow-billed Duck genotype class across all five individuals: $\bar{x} = 0.986$). Natural Yellow-billed Duck populations (Strandfontein and Barberspan) had a high mean posterior probability of being assigned as pure individuals ($\bar{x} = 0.972$). However, eight hybrid individuals were also identified in these populations.

Our simulation results supported the genotype classes determined by NEWHYBRIDS. When applying the confidence intervals from the simulated data to the actual data, no indiviuals fell within the 95% confidence interval calculated for F1 assignment values (q_{ik}) for assignment to the Mallard Duck cluster, and only one individual fell within the 95% confidence interval for F2 assignment values (q_{ik}) (Figure S5). Additionally, the simulation indicated that it is difficult to distinguish between F1 and F2 individuals, as these have overlapping confidence

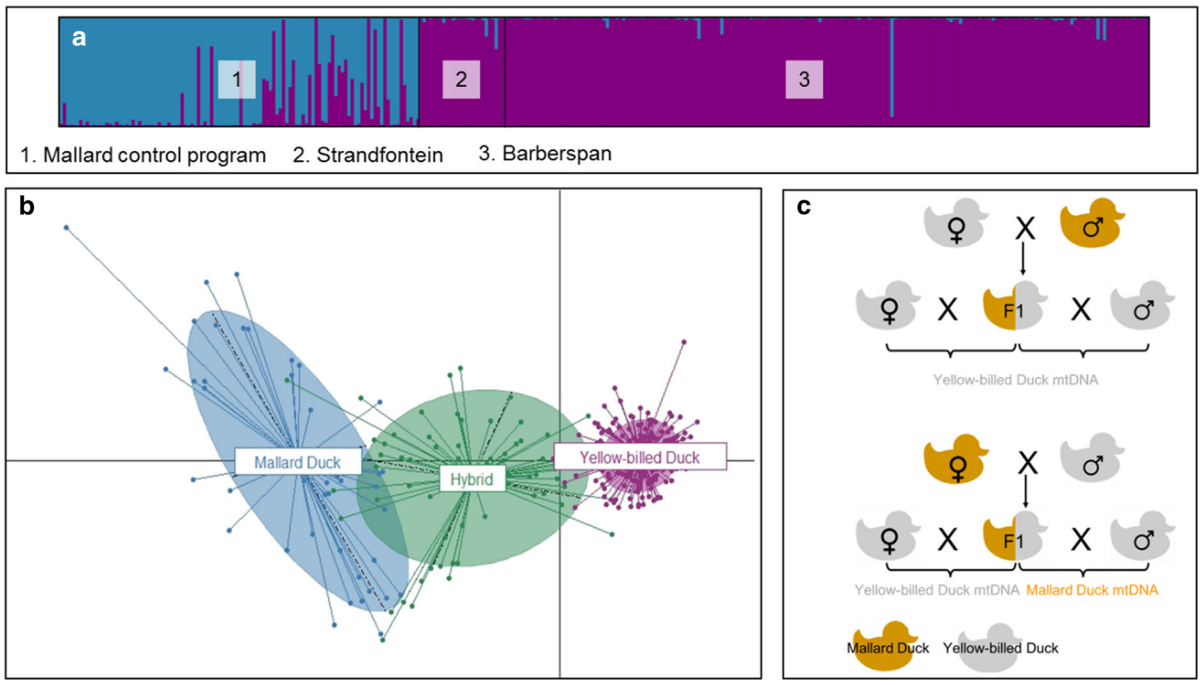

Fig. 2 **a** Scatter plot of principal components analysis showing genetic structure between Mallard Ducks (blue), Yellow-billed Ducks (purple) and putative hybrids (green) (PCA axis 1 = 9.0%; PCA axis 2 = 3.4%). **b** Structure bar plot where each bar represents an individual and the colour of the bar indicates the proportion of assignment to each genetic cluster (K = 2), Mallard Duck (blue) and Yellow-billed Duck (purple) respectively. The individuals are organised by population. (c) Diagram illustrating two possible crosses that can result in Yellow-billed Duck backcrosses. Mallard Duck and Yellow-billed Duck mitochondrial DNA (mtDNA) haplotypes are indicated as orange and grey respectively. Only one possible combination of crosses can result in a backcrossed Yellow-billed Duck with a Mallard Duck mtDNA haplotype

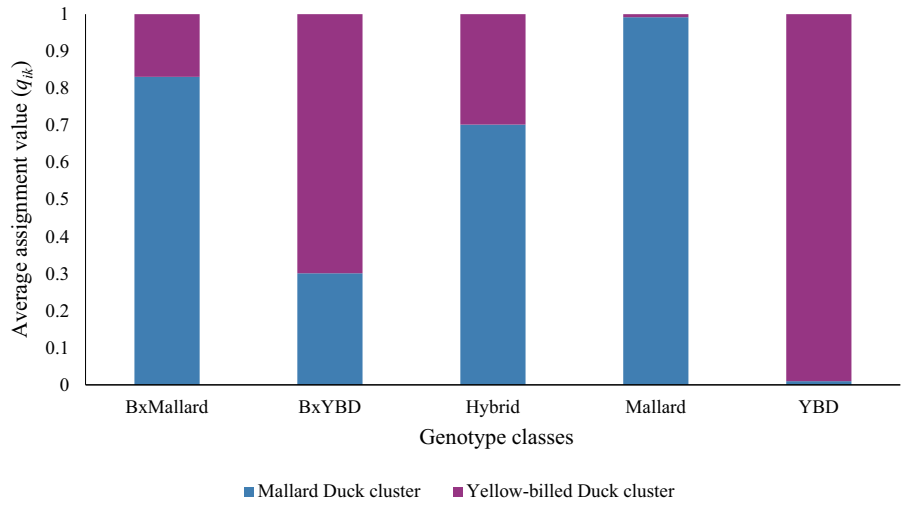

Fig. 3 A bar plot illustrating the average assignment value (q_{ik}) from STRUCTURE to each cluster [Mallard Duck (in blue) and Yellow-billed Duck (in purple)] for all the individuals in each genotype class classified by NEWHYBRIDS [BxMallard (Mallard Duck backcross, n = 15); BxYBD (Yellow-billed Duck backcross, n = 5); Hybrid (Hybrid of mixed ancestry, n = 47); Mallard (n = 46); YBD (Yellow-billed Duck, n = 220)]

intervals, a common limitation associated with microsatellite analyses (e.g. Le Roux et al. 2015). The majority of the samples fell outside the confidence intervals for F1 and F2 hybrids, supporting extensive backcrossing between hybrids and parental species.

Assessing directionality of Yellow-billed/Mallard Duck introgression and sex-biased mating

Gene flow from Mallard Ducks into Yellow-billed Duck populations was estimated to be 0.0019 migrants per generation (95% credible interval − 0.002 to 0.031), while gene flow in the opposite direction was significantly higher (0.0129 migrants per generation, 95% credible interval − 0.005 to 0.005; Wilcoxon signed-rank test, $p < 0.001$, Z = 6073, n = 1000 MCMC samples).

Two species-specific synapomorphies within the ND2 mtDNA region differentiated Mallard from Yellow-billed Ducks. All hybrid individuals that were sequenced carried the Mallard Duck ND2 haplotype (Fig. 4; Table S3). Similarly, based on ND2 haplotypes, three individuals that were classified as Yellow-billed Duck backcrosses must have resulted from a cross between a Mallard Duck hen × Yellow-billed Duck drake, and subsequent backcross with a Yellow-billed Duck drake (Fig. 2c). For the remaining hybrids, where we were unable to classify them into specific hybrid genotype classes, we cannot be certain whether the original parents followed the same parental contributions as for Yellow-billed Duck backcrosses. Given that all sequenced samples were carriers of the Mallard Duck ND2 haplotype, it is reasonable to assume that most hybridisation events involve Mallard Duck hens.

Discussion

We found low population genetic differentiation between three widely distributed Yellow-billed Duck populations in South Africa. We therefore conclude that high levels of gene flow maintain similar levels of genetic diversity within these populations. The high levels of gene flow are most likely due to frequent long-distance dispersal of the species, since Yellow-Billed Ducks are highly mobile with bird ringing and recapture records suggesting that dispersal can sometimes exceed 1000 km (Underhill et al. 1999). Other highly dispersive duck species like the Mottled Duck (*Anas fulvigula*), the White-headed Duck (*Oxyura leucocephala*), the Northern Pintail (*Anas acuta*), and King Eiders (*Somateria spectabilis*) generally show similarly low population genetic structure throughout large portions of their ranges (Williams et al. 2002; Pearce et al. 2004; Muñoz-Fuentes et al. 2005; Flint et al. 2009).

Our study also provides the first scientific evidence for the occurrence of hybridisation between invasive Mallard and native Yellow-billed Ducks in South Africa. However, contrary to our expectations, we found little introgression into Yellow-billed Duck populations, but rather that the direction of gene flow was into the Mallard Duck gene pool. The latter was supported by the identification of sex-biased mating, whereby most hybrids in our populations resulted from crosses between Mallard Duck hens and Yellow-billed Duck drakes. This, together with the lack of population structure found between widely distributed South African Yellow-billed Duck populations suggests that hybrid or introgressed genotypes may reach far-off Yellow-billed Duck populations through long-distance dispersal. This can lead to an intriguing phenomenon whereby genes of non-native Mallard Ducks can spread through native populations, even in the absence of Mallard Ducks. Notably, this indicates that Mallard control needs to be conducted at a national level because it is likely that hybridisation can

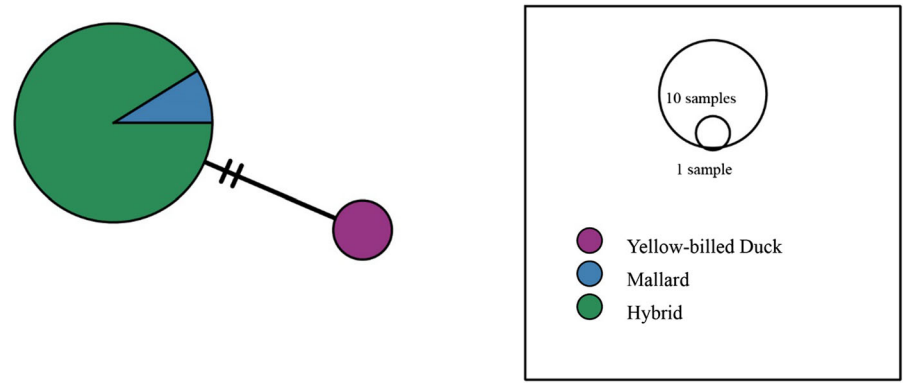

Fig. 4 A haplotype network illustrating the two ND2 mtDNA haplotypes: Mallard Duck (in blue) and Yellow-billed Duck (in purple). All hybrid individuals (in green) that were sequenced were found to contain the Mallard Duck haplotype

spread through these movements. It is important to note that we only sampled a limited number of Yellow-billed Duck populations and further sampling throughout the species' African range is needed to determine the extent of panmixia in this species.

Overall, our findings provide a positive outlook for the long-term genetic integrity of Yellow-billed Ducks in South Africa. Introgression of Mallard Duck genes into Yellow-billed Duck populations appears to be limited (only 2.3% Yellow-billed Duck backcrossed individuals were detected), and instead occurs primarily into the Mallard Duck gene pool. Our findings are in contrast to most other known instances of hybridisation between Mallard Ducks and other *Anas* species from elsewhere in the world (Rhymer et al. 1994; Mank et al. 2004; Williams et al. 2005; Fowler et al. 2009). In most of these cases significant hybridisation and introgression has occurred between Mallard Ducks and native ducks. For example, there is a high degree of hybridisation and introgression between Mallard Ducks and American Black Ducks (*Anas rubripes*, Mank et al. 2004), New Zealand Grey Ducks (*A. superciliosa*, Rhymer et al. 1994), and Mottled Ducks (*A. fulvigula*) (Williams et al. 2005). Despite the limited introgression into Yellow-billed Duck populations in South Africa, we would recommend follow-up studies to ascertain whether the patterns we found here hold up across the species' range or change over time. Compared to South Africa, Mallard Ducks have longer residence times in countries where introgression is extensive. For example, Mallard Ducks were introduced to New Zealand in the late 1800s (Dyer and Williams 2010) and are native to the western United States, having expanded their range eastward in the late 1800s (Johnsgard 1961; Brodsky and Weatherhead 1984; Heusmann 1991). Mallard Ducks have only established relatively recently in South Africa (1940s) and this, coupled with large resident Yellow-Billed Duck populations (ca. 65,000 individuals; Rowan 1963; Geldenhuys 1975; Skead and Dean 1977), may relate to low levels of hybridisation.

Although levels of hybridisation between Mallard and Yellow-billed Ducks in South Africa appears to be limited, we did find evidence for the occurrence of backcrossing. A low number of F1 and F2 individuals were also identified in our dataset and most hybrids could not be assigned to specific genotype classes (i.e. F1, F2, or backcrossed) with high levels of certainty in our analyses and were classified as hybrids of 'mixed ancestry'. This suggests that most hybrids represent several generations of backcrossing, rather than F1 hybrids. This might be expected if F1 hybrids are the result of unusual events, such as the introduction of Mallard Ducks to large Yellow-billed Duck assemblages, or if early control measures removed all F1 hybrids. We also found some hybrid individuals, albeit a few, in Yellow-billed Duck populations. Furthermore, we determined that in some instances putative 'hybrids' were indeed pure Yellow-billed Ducks illustrating the need for continuous genetic monitoring to ensure that eradication programs do not target native ducks. These findings also suggest that general morphological characters, like feet pigmentation, used in this study are inadequate to identify putative hybrids. However, although these Yellow-billed Ducks were potentially euthanised unnecessarily, the remaining population is large and widespread (Rowan 1963; Geldenhuys 1975; Skead and Dean 1977; South African Bird Atlas Project 2 2019b), so removing a few individuals will not have a major impact on the population. Furthermore, it is better to remove individuals that could be "pure" Yellow-billed Ducks than risk not removing potential hybrids. This is a real risk considering that backcrossed individuals are often morphologically indistinguishable from parental species (Rhymer and Simberloff 1996; Devillard et al. 2014).

Given the reproductive behaviour and ecology of Mallard Ducks, we expected to find sex-biased mating between Mallard Ducks and Yellow-billed Ducks, with most hybrids resulting from mating between Yellow-billed Duck hens and Mallard Duck drakes. Specifically, Mallard Duck drakes are known to be aggressive maters (Seymour 1990; D'Eon et al. 1994), and they are able to outcompete congeneric drakes for available hens (e.g. American Black Ducks, Brodsky and Weatherhead 1984). In contrast, we found most hybrids to have resulted from mating between Yellow-billed Duck drakes and Mallard Duck hens, a finding that corroborates observations in the Western Cape Province (Western Cape Birding 2013). There is also observational evidence for mating to occur between Mallard Duck drakes and Yellow-billed Duck hens, but our genetic evidence suggests that these events are uncommon. Similar sex-biased mating between Mallard Duck hens and drakes of other *Anas* species has been previously shown (Rhymer et al. 1994; Kulikova

et al. 2004; Fowler et al. 2009). These studies, together with our data, suggest that the assumption that Mallard Duck drakes are the primary initiators of hybridisation with closely related *Anas* species is not warranted. Sex-biased mating between Yellow-billed and Mallard Ducks also supports the directionality of introgression we inferred from our microsatellite data. If most hybridisation occurs between Mallard Duck hens and Yellow-billed Duck drakes, then ducklings are likely to be raised within the Mallard Duck population (Steyn 1996; Doherty et al. 2002). Field observations indicated that Mallard and Yellow-billed Ducks tend to remain in separate populations, making it likely that hybrid offspring would have limited contact with Yellow-billed Ducks and therefore hybrid offspring are more likely to mate with Mallard Duck individuals within their own populations (Kruijt et al. 1982). Another intriguing possibility is that female F1 hybrids are less viable, or even infertile, compared to male F1 hybrids. This would be in line with the frequent observation of Haldane's rule: that when only one gender of hybrids is inviable in species crosses, it is usually the heterogametic sex (females in the case of birds; Haldane 1922). This would mean that backcrosses predominantly involve F1 males and pure females. Our analyses found backcrossed individuals to always carry Mallard mtDNA haplotypes (see Table S3), and together with our nuclear microsatellite analyses, suggest that pure Mallard Duck females are predominantly involved in these backcrosses. In support of Haldane's rule, Kirby et al. (2004) found American Black Duck and Mallard Duck crosses to produce predominantly male offspring (65% of total), while sex ratios of backcrossed and F2 hybrids approached 1:1. Interestingly, this also seems to hold up among domestic races of Mallard Ducks (Phillips 1914). Whether Haldane's rule applies to Yellow-billed × Mallard Duck crosses in South Africa deserves futher research attention.

Our findings paint a positive picture for the genetic integrity of South African Yellow-billed Ducks in the long term. Current populations in Cape Town and Barberspan (and likely the rest of South Africa) represent a panmictic population, precluding the need to focus conservation efforts on genetically unique subpopulations. Additionally, hybridisation with the Mallard Duck appears limited with low levels of introgression into the Yellow-billed Duck gene pool. These findings suggest that long-term impacts on the evolutionary potential of Yellow-billed Ducks, e.g. the purging of adaptive genetic variation, appears to be negligible at this point in time. However, introgression could become more extensive in the future. Moreover, there is potential for hybridisation to spread through long distance movements and we do not know the extent of hybridisation and introgression in other parts of South Africa, or the rest of the Yellow-billed Ducks' range. It is thus advisable to remove Mallard Ducks and their hybrids while such interventions will have a high chance of success. That is, unlike other native *Anas* species impacted by Mallard Duck hybridisation around the world, there is still a good chance of protecting the long-term genetic integrity of South Africa's Yellow-billed Duck population through the removal of Mallard Ducks and their hybrids at a national level. Our hope is that when this message is communicated effectively to the communities where Mallard Duck removal efforts continue, that it will sway the public's perception in favour of the control of this invasive duck. We also strongly advise that future work should include other native *Anas* species in South Africa to ascertain whether hybridisation and introgression is similarly restricted as in the case of Yellow-billed Ducks.

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