

# Genetic structure among *Charadrius* plovers on the African mainland and islands of Madagascar and St Helena

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Colonization of islands by long-distance dispersers has great impact on genetic diversification among populations and may spearhead speciation events. We investigated intra- and interspecific divergence in *Charadrius* plovers with populations on mainland Africa, Madagascar and St Helena. We analysed microsatellite loci and sequence data from four nuclear and two mitochondrial gene regions. *Charadrius* plovers are shorebirds with high dispersal and mobility. Our results confirmed genetic differentiation between Madagascar and mainland populations of three plover species (White-fronted Plover *Charadrius marginatus*, Kittlitz's Plover *Charadrius pecuarius* and, based on sequence data only, Three-banded Plover *Charadrius tricollaris*) but highlight substantial variation in levels of intraspecific divergence among the three species. Namely, the Kittlitz's Plover, a dispersive habitat generalist with a polygamous mating system, exhibited lower island–mainland differentiation (0.05% COI sequence divergence) compared with the two monogamous species, the White-fronted Plover (0.6% COI divergence) and Three-banded Plover (1.6% COI divergence). In addition, past colonization of the islands of St Helena and Madagascar by ancestors of today's Kittlitz's Plover has led to the evolution of two endemic island species, the Madagascar Plover *Charadrius thoracicus* and the more closely-related St Helena Plover *Charadrius sanctaehelenae*. We discuss the factors driving species differences in island–mainland divergence and highlight the importance of conserving genetically unique island populations and island habitats to safeguard future evolutionary potential.

**Keywords:** dispersal, population genetics, speciation, waders.

Islands have long been known to have a profound impact on the formation and diversity of species, a point that inspired Darwin (1845) concerning the mechanisms of evolution and led to the emergence of an entire discipline: island biogeography (MacArthur

& Wilson 1967, Warren *et al.* 2015). Islands are particularly important in speciation for highly mobile species such as birds, for which mainland habitats often provide no barriers to dispersal. Mainland populations of these species remain highly connected and genetically homogeneous, meaning that the emergence of genetic differentiation, the precursor of speciation, is hindered (Ibrahim *et al.* 1996, Phillimore *et al.* 2006, Agnarsson & Kuntner 2012). For

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example, mainland populations of Kentish Plover *Charadrius alexandrinus* across continental Eurasia are genetically similar up to 10 000 km apart, with divergence observed only among populations isolated by oceanic barriers (Küpper *et al.* 2012). Furthermore, populations that successfully colonize smaller, more distant islands are expected to develop greater genetic divergence from mainland populations compared with those occupying larger islands closer to the mainland (Amos & Harwood 1998).

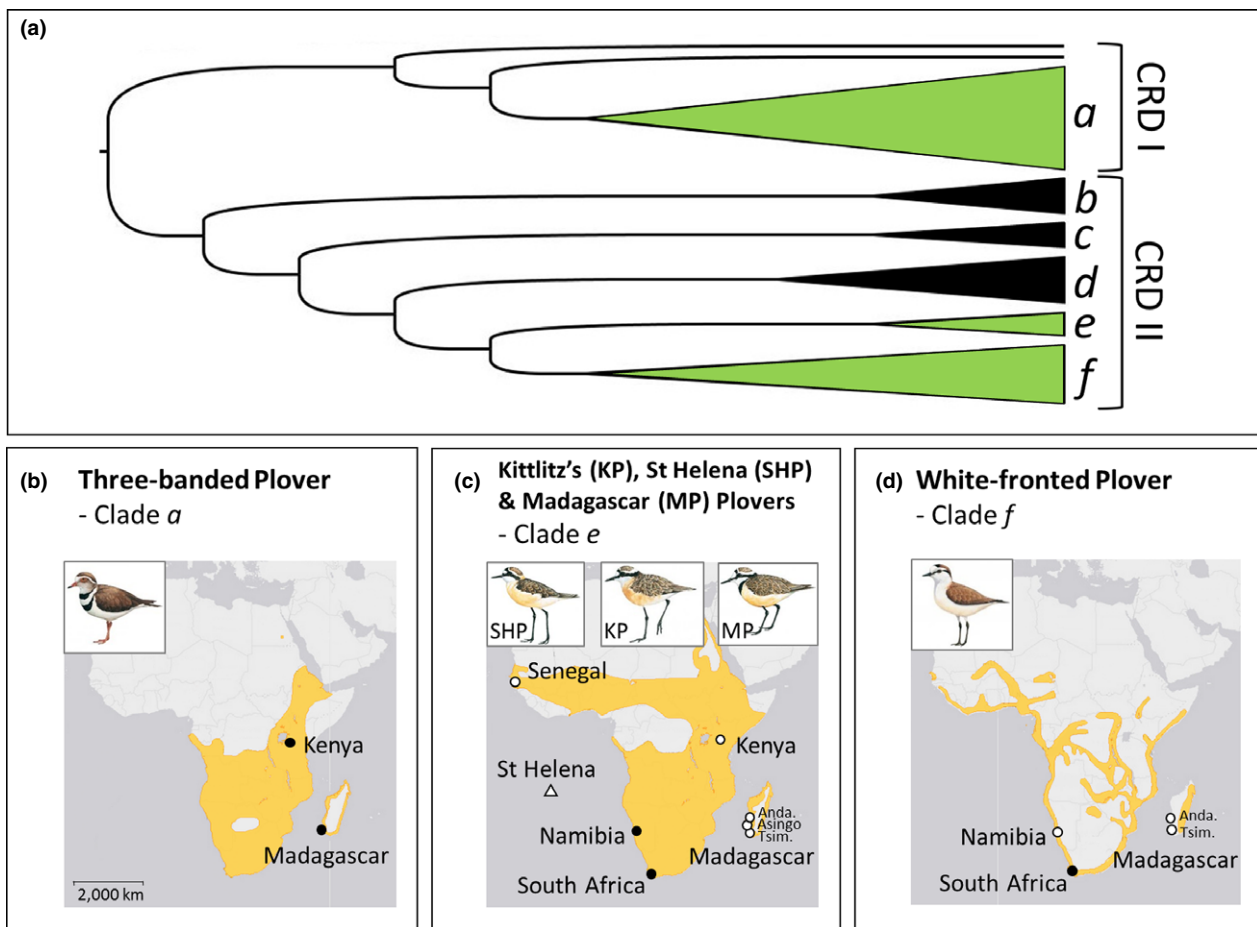
To investigate further the role of islands in promoting genetic differentiation and speciation in avian populations, we focused on the small plovers, genus *Charadrius* (c. 31 species; Linnaeus 1758). Plovers are shorebirds capable of traversing large distances, and several species are migratory (Stenzel *et al.* 1994, Hedenström *et al.* 2013). They have precocial offspring and are of interest in evolutionary biology for their highly variable mating systems (Thomas *et al.* 2007). Nine *Charadrius* species breed in Africa, including three species with populations on both Madagascar and the mainland. Previously we assigned these African species to three distinct phylogenetic clades (see Fig. 1a) that independently colonized the continent following dispersal southwards from Palaearctic, Saharo-Arabian and Central Asian regions (dos Remedios *et al.* 2015). Each of the three clades includes one species with populations on both Madagascar and the mainland: the Three-banded Plover *Charadrius tricollaris* (Clade a), the Kittlitz's Plover *Charadrius pecuarius* (Clade e) and the White-fronted Plover *Charadrius marginatus* (Clade f; see Fig. 1 for species distributions). Levels of genetic divergence among these populations have not previously been examined and comparison between species may reveal insights into the development of genetic differentiation, and ultimately speciation.

Among the African small plovers are two island endemics, one species on the island of St Helena and the other on Madagascar: the St Helena Plover *Charadrius sanctaehelenae* and Madagascar Plover *Charadrius thoracicus* (Hayman *et al.* 1986). Madagascar is a large island, 587 040 km<sup>2</sup> in size, separated from mainland Africa by 400 km. At 420 km<sup>2</sup> in size, St Helena is a much smaller and far more isolated island, lying 1950 km from mainland Africa. The St Helena Plover and Madagascar Plover are close sister species to the widespread Kittlitz's Plover, representing two of the most recent cases of speciation within the genus *Charadrius* (dos Remedios *et al.* 2015). The St Helena Plover is approximately 30% larger than the

Kittlitz's Plover and exhibits reduced tan coloration on the breast. In contrast, the Madagascar Plover is distinguished from both sister species by a strong black breast band (del Hoyo *et al.* 1996, Rowlands *et al.* 1998). The conservation status of both endemics is 'vulnerable' and populations are at risk from habitat loss as well as introduced predators such as cats and rats (Birdlife International 2016a,b). Until recently, the St Helena Plover population was in decline and was classified as 'critically endangered' (McCulloch 2009), but successful management of its grassland breeding areas has prompted an increase in population size, from approximately 220 adults in 2006 to approximately 560 in 2016 (BirdLife International 2016a, b). The Madagascar Plover has a larger population of approximately 2000 individuals, but this population is currently thought to be in decline due to the destruction of habitats on the west coast of Madagascar (Birdlife International 2016b). Both species are habitat specialists, breeding in areas of short-sward grassland, making them particularly vulnerable to habitat loss.

In this study, we first examined population-level genetic divergence among African *Charadrius* plovers, with a focus on island-mainland differentiation, in three species with breeding populations on both mainland Africa and Madagascar. For two species, the Kittlitz's Plover and the White-fronted Plover, we assessed population divergence using both microsatellite data and sequence-based (nuclear and mitochondrial) analyses. For a third species, the Three-banded Plover, microsatellite analyses were not possible due to a limited sample size on mainland Africa (Kenya,  $n = 1$ ), and therefore we conducted sequence-based analysis only. We predicted that the connectivity of plover populations would be disrupted by oceanic barriers, and hence island populations should be clearly genetically differentiated from their mainland counterparts, whereas high connectivity among continental populations should lead to lower genetic differentiation.

Additionally, we used microsatellite and sequence-based analyses to examine genetic divergence between populations of the Kittlitz's Plover and its two closely related endemic sister species, the St Helena Plover and Madagascar Plover. Based on island size, population size and distance to the mainland, we expected that Kittlitz's Plovers would exhibit a greater degree of divergence from St Helena Plovers than from Madagascar Plovers. Lastly, for all populations assessed using



**Figure 1.** (a) An overview of phylogenetic relationships within the genus *Charadrius* (major clades *CRD I* and *CRD II*, minor clades *a–f*, based on dos Remedios *et al.* 2015). Highlighted clades (*Clades a, e* and *f*) include the African study species. (b–d) Distribution maps for the three endemic African species in population-level genetic analyses, including breeding ranges (shaded areas) and sampling locations (labelled symbols). Open circles indicate populations included in microsatellite analyses (Fig. 2); closed circles indicate populations assessed based on nuclear and mitochondrial sequence data only. An open triangle indicates the location of St Helena Plovers, analysed at microsatellite loci alongside Madagascar Plovers and Kittlitz's Plovers (all three sister species within *Clade e*). Sampling sites in Madagascar were at Andavadoaka (Anda.), Asingo and Tsimanampetsotse (Tsim.). Madagascar Plovers were sampled at Tsimanampetsotse. Distribution maps are adapted from those provided by The IUCN Redlist of Endangered Species (BirdLife International & NatureServe 2014). Images by Wiersma *et al.* (2016a,b,c).

microsatellite data, we evaluated coefficients of inbreeding, with the expectation that levels of inbreeding would be greater for island than for mainland populations.

## METHODS

### Taxon sampling and DNA extraction

Samples were collected from five plover species (sample sizes listed in Table 1; distribution maps in Fig. 1). For each sample, 25–50  $\mu$ L of blood was collected and stored in 1 mL Queen's Lysis

Buffer (Seutin *et al.* 1991) or 1 mL 95% ethanol. DNA was extracted from blood samples using an ammonium acetate precipitation method (Nicholls *et al.* 2000, Richardson *et al.* 2001).

Genotyping at microsatellite loci was carried out for Madagascar and mainland African populations of two African endemic species, the White-fronted Plover and Kittlitz's Plover, as well as for two close sister species of the latter, the Madagascar Plover and St Helena Plover (Table 1). Analyses of sequence data were performed for all species and sampling sites, including both Madagascar and mainland populations of the White-fronted

**Table 1.** Sampling locations and sample sizes for microsatellite analyses of plover species (genus *Charadrius*). Initial sample sizes are those prior to, and final sample sizes are those after the exclusion of samples with high relatedness ( $R > 0.5$ ) based on microsatellite data. For sequence-based analyses, three individuals were analysed per location in all cases except for the Kittlitz's Plovers of Namibia ( $n = 2$ ), White-fronted Plovers of South Africa ( $n = 1$ ) and Three-banded Plovers of Kenya ( $n = 1$ ). Madagascar sampling sites were at Andavadoaka (Anda.), Asingo and Tsimanampetsotse (Tsim.). See Figure 1 for mapped locations and species distributions.

Species	Sampling location	Initial sample size	Final sample size	% Samples excluded
Kittlitz's Plover, <i>Charadrius pecuarius</i>	Kenya <sup>M</sup>	28	19	32
	Senegal <sup>M</sup>	14	10	29
	Anda. (Madagascar) <sup>I</sup>	30	21	30
	Asingo (Madagascar) <sup>I</sup>	11	9	18
	Tsim. (Madagascar) <sup>I</sup>	29	21	28
	South Africa <sup>M,a</sup>	—	—	—
	All mainland	42	29	31
	All island	70	51	27
St Helena Plover, <i>Charadrius sanctaehelenae</i>	St Helena <sup>I</sup>	31	11	65
Madagascar Plover, <i>Charadrius thoracicus</i>	Tsim. (Madagascar) <sup>I</sup>	31	18	42
White-fronted Plover, <i>Charadrius marginatus</i>	Namibia <sup>M</sup>	20	19	5
	Anda. (Madagascar) <sup>I</sup>	30	19	37
	Tsim. (Madagascar) <sup>I</sup>	32	25	22
	South Africa <sup>M,a</sup>	—	—	—
	All mainland	20	19	5
	All island	62	44	29
	Kenya <sup>M,a</sup>	—	—	—
Three-banded Plover, <i>Charadrius tricollaris</i> <sup>a</sup>	Tsim. (Madagascar) <sup>I,a</sup>	—	—	—

<sup>M</sup>Mainland locations; <sup>I</sup>island locations; <sup>a</sup>samples included in sequence-based analyses only.

Plover, Kittlitz's Plover and Three-banded Plover (Table 1).

### Microsatellite genotyping

Utilizing microsatellite primers largely developed for the genus *Charadrius* (see Tables S2 and S3), we initially tested markers in eight Kittlitz's Plover and eight White-fronted Plover samples. We selected polymorphic loci for which at least two different alleles were detected and designed multiplexes using MULTIPLEX MANAGER v1.2 (Holleley & Geerts 2009) for these two species separately. Multiplexes designed based on Kittlitz's Plover samples were also used for analyses of its sister species, the St Helena Plover and Madagascar Plover. We conducted polymerase chain reactions (PCRs) in 2- $\mu$ L volumes, including each primer at 0.2  $\mu$ M (forward primers labelled with fluorescent dye) and 1  $\mu$ L Qiagen Multiplex PCR MasterMix. Each reaction was sealed with mineral oil to prevent evaporation. PCR amplification was conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) as follows: 95 °C for 15 min, 35 cycles of 94 °C for

30 s, 56 °C for 90 s, 72 °C for 60 s, and a final extension of 60 °C for 30 min. Amplicons were visualized on an ABI 3730 automated DNA analyser and fragment lengths were scored using GeneMapper software version 4.1 (Applied Biosystems, Waltham, MA, USA). For 5% of samples, PCR amplification and scoring were repeated a second time in a blind test. Genotyping was consistent across replicates in all cases.

Sampled individuals in each population were putatively unrelated, with known parent-offspring and sibling pairs excluded. To further avoid inadvertent sampling of highly related individuals, levels of genetic relatedness among individuals were evaluated in MLRelate (Kalinowski *et al.* 2006) and those with relatedness of  $R > 0.5$  (corresponding to full sibling or parent-offspring relatedness; Blouin 2003) were removed from further analyses. Removed samples included 19 of 82 White-fronted Plovers, 32 of 112 Kittlitz's Plovers, 20 of 31 St Helena Plovers and 13 of 31 Madagascar Plovers (for final sample sizes per population see Table 1).

Deviations from Hardy-Weinberg equilibrium at each marker were assessed in CERVUS v3.0.3



(Kalinowski *et al.* 2007) and markers were excluded from further analyses where deviations occurred and null allele frequencies were high ( $> 0.2$ ) in two or more populations. For Kittlitz's Plovers, St Helena Plovers and Madagascar Plovers, nine markers were included in the final set; for White-fronted Plovers, 17 markers remained (see marker sets in Tables S2 and S3).

### Microsatellite-based population analyses

To determine relative levels of genetic diversity for each marker and species, allelic richness and inbreeding coefficients ( $F_{IS}$ ) were estimated in FSTAT 2.9.3 (Goudet 1995), and global fixation indices ( $F_{ST}$ ,  $G'_{ST}$  and  $D$ ) per species were estimated using the 'diveRsity' package in R ('diffCalc' function; Keenan *et al.* 2013). To evaluate genetic differentiation among populations of Kittlitz's Plovers and White-fronted Plovers, ARLEQUIN 3.5.2 (Excoffier & Lischer 2010) was used to estimate pairwise fixation indices ( $F_{ST}$ ) and perform analysis of molecular variance (AMOVA). Using AMOVA, we assessed all possible population groupings of these two species to determine which arrangement maximized molecular variance between as opposed to within groups.

We additionally assessed genetic structure among populations using principal component analysis (PCA; 'adeigenet' package in R (v3.0.3; R Core Team 2013)) and STRUCTURE 2.3.4 (Pritchard *et al.* 2000, Hubisz *et al.* 2009). Both programs were implemented for the White-fronted Plover, the Kittlitz's Plover along with the St Helena Plover and Madagascar Plover, and the Kittlitz's Plover alone. STRUCTURE analyses were carried out to determine the number of genetic clusters ( $K$ ) within each sample set. An admixture model was utilized, with independent allele frequencies, for 500 000 Markov chain Monte Carlo (MCMC) generations after a burn-in of 100 000.  $K$  values of 1–4 were assessed for the White-fronted Plover,  $K$  values of 1–7 for the Kittlitz's Plover, St Helena Plover and Madagascar Plover, and  $K$  values of 1–5 for the Kittlitz's Plover-only analyses. To determine the convergence of MCMC runs, convergence of the parameter alpha (indicating relative admixture between populations) was assessed throughout each run. STRUCTURE analyses were repeated twice for each dataset and results (and alpha convergence) were consistent

between repeats. Results were evaluated using STRUCTURE HARVESTER (Earl & vonHoldt 2012) and plots were constructed with CLUMPP (Jakobsson & Rosenberg 2007) and DISTRICT (Rosenberg 2004).

### Sequence analyses

Six genes were targeted for PCR amplification and sequencing including four nuclear loci: *ADH5* (alcohol dehydrogenase 5; 829-bp partial coding sequence; Fain *et al.* 2007), *FGB7* ( $\beta$ -fibrinogen intron 7; 840 bp; Prychitko & Moore 1997), *MYO2* (myoglobin intron 2; 688 bp; Slade *et al.* 1993) and *RAG1* (recombination activating gene 1; 911-bp partial coding sequence; Groth & Barrowclough 1999); and two mitochondrial loci: *COI* (cytochrome oxidase I; 626 bp; Hebert *et al.* 2004) and *ND3* (NADH dehydrogenase subunit 3; 401 bp; Chesser 1999). Sequences for one population per species were the same as those utilized by dos Remedios *et al.* (2015) (see Table S1 for details). For full details of primers, PCR and sequencing conditions see dos Remedios *et al.* (2017).

Sequences were edited and aligned in Codon-Code Aligner 3.7.1 (CodonCode Corporation, Centerville, MA, USA) using the ClustalW algorithm, and sequence data were deposited in the GenBank sequence database (for accession numbers see Table S1). DNAsp v5 (Librado & Rozas 2009) was used to assess nucleotide divergence across sampled populations.

## RESULTS

### Population-level island–mainland genetic differentiation

Clear genetic differentiation was present between Madagascar and mainland Africa for all three species assessed at the population level, although the degree of differentiation varied among species. Specifically, populations of White-fronted Plovers and Three-banded Plovers exhibited greater island–mainland differentiation than did populations of Kittlitz's Plovers (as described below). For White-fronted Plovers and Kittlitz's Plovers, this pattern was evident in global fixation indices based on microsatellite data: White-fronted Plover  $F_{ST} = 0.367$  and Kittlitz's Plover  $F_{ST} = 0.029$ . Three-banded Plovers were not evaluated due to low sample size.

### White-fronted Plovers

Based on microsatellite (allele size) data, the White-fronted Plovers of Madagascar (two sites) represented a genetic cluster distinct from the Namibian population (3400 km away). Of the microsatellite variance, 46.13% was attributed to differences between these clusters based on AMOVA (Table 2; see Table 3 for pairwise fixation indices ( $F_{ST}$ )) and these clusters were identified based on both STRUCTURE analyses (Fig. 2a;  $K = 2$ ,  $\Delta K = 4509.27$  vs.  $K = 3$ ,  $\Delta K = 0.81$ ; for full  $K$ -value comparison see Fig. S1 and Table S4) and PCA (see Fig. 2a).

Madagascar and mainland (Namibian and South African) populations exhibited moderate genetic divergence based on mitochondrial and nuclear sequence data (0.9% mitochondrial divergence; 0.2% nuclear divergence; Table 4) and genetic differentiation also emerged among mainland (Namibian and South African) White-fronted Plover populations, although the overall degree of divergence was lower and divergence was present in mitochondrial sequence data only (0.4% mitochondrial divergence; 0% nuclear divergence; Tables 4 and S5).

### Kittlitz's Plovers

Kittlitz's Plovers on Madagascar and mainland Africa (Kenya and Senegal) formed two genetic clusters based on microsatellite loci, albeit with weaker differentiation than for White-fronted Plovers. Only 3.73% of molecular variance emerged between Madagascar and mainland populations of the Kittlitz's Plover based on AMOVA (Table 2; see Table 3 for pairwise fixation indices ( $F_{ST}$ )) and both PCA and STRUCTURE analyses concurred

in identifying weak divergence between clusters (Fig. 2b;  $K = 2$ ,  $\Delta K = 331.94$  vs.  $K = 3$ ,  $\Delta K = 16.99$ ; for full  $K$ -value comparison see Fig. S1 and Table S4). Between the two mainland populations in Kenya and Senegal, there was no identifiable genetic structure in the microsatellite data (Fig. 2b). These results were qualitatively the same whether we included or excluded the two sister species, the St Helena Plover and Madagascar Plover, in this analysis (Fig. 2b,c).

Based on mitochondrial sequence data, Kittlitz's Plover populations exhibited extremely low diversity between Madagascar and mainland populations (< 0.1% divergence; Tables 4 and S5) and also among mainland sites (0.1% mitochondrial divergence). In comparison, at nuclear loci, greater overall sequence diversity was present in terms of the number of polymorphic sites ( $S$ ; Tables 4 and S5), but divergence between populations was again low, with 0.2% nuclear divergence between Madagascar and mainland populations and 0.1% divergence among mainland sites.

### Three-banded Plovers

The Three-banded Plovers of Madagascar and Kenya displayed moderate genetic differentiation based on mitochondrial sequence data (1.1% divergence) and low divergence at the four nuclear loci (0.04% divergence; Tables 4 and S5). This species was not assessed at microsatellite loci due to low sample size.

### Species divergence on Madagascar and St Helena

Regarding the genetic similarity between the Kittlitz's Plover and its close sister species, the

**Table 2.** Analysis of molecular variance (AMOVA) for best groupings of two African plover species, the White-fronted Plover (Namibia distinct from two Madagascar sites) and Kittlitz's Plover (mainland sites in Kenya/Senegal distinct from three Madagascar sites). These groupings were those with the highest 'among group' variance based on separate AMOVAs for each possible grouping of populations.

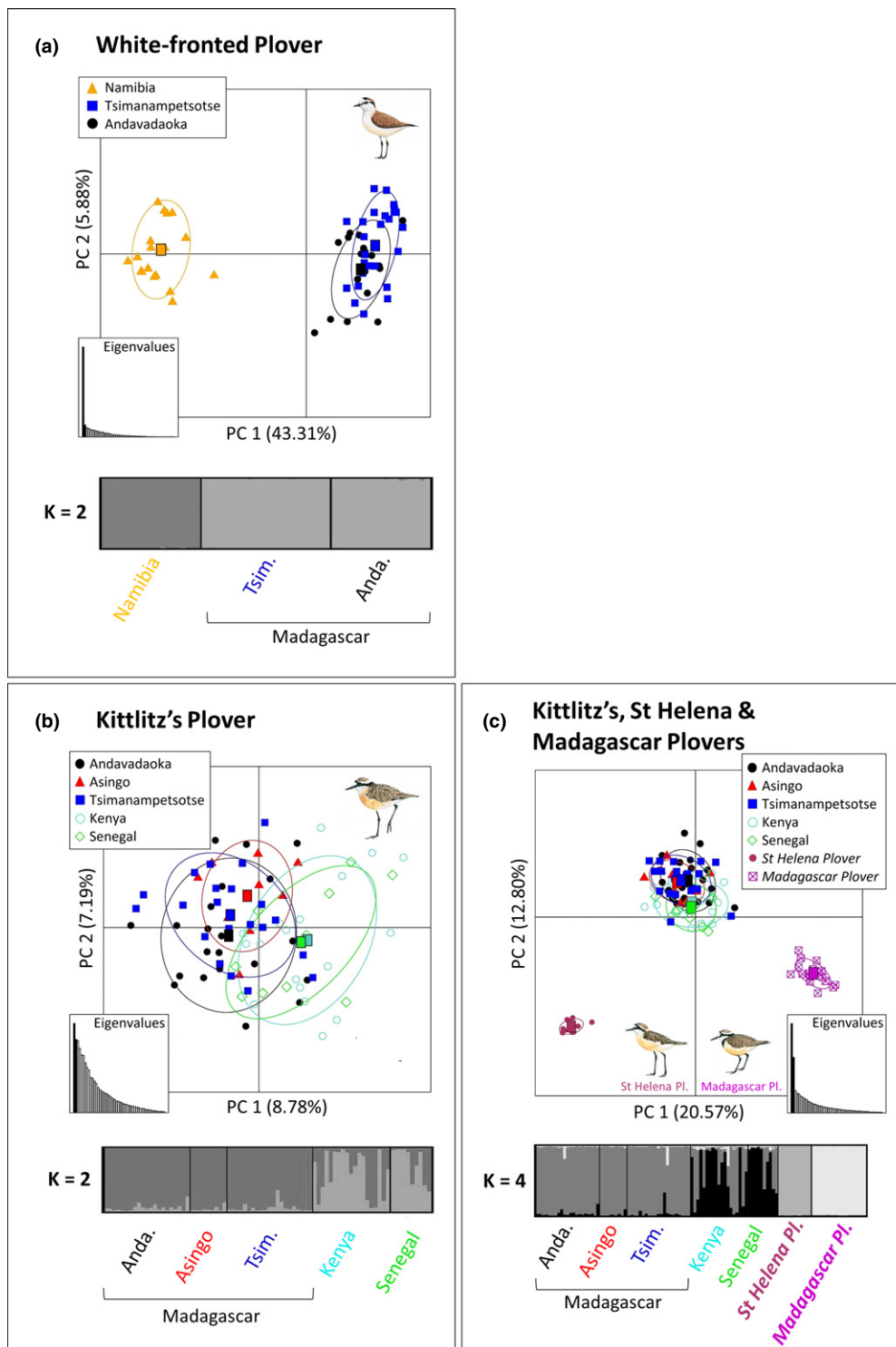
	White-fronted Plover				Kittlitz's Plover			
	df	SS	Va	%	df	SS	Va	%
Among groups	1	131.0	2.38	46.13	1	8.8	0.08	3.73
Among populations within groups	1	5.0	0.05	1.01	3	7.6	0.01	0.53
Within populations	123	335.1	2.72	52.86	155	335.7	2.17	95.74
Total	125	471.1	5.15		159	352.1	2.26	

df, degrees of freedom; SS, sum of squares; Va, variance; %, percentage of total variance.

**Table 3.** Pairwise  $F_{ST}$  values based on microsatellite data among populations of Kittlitz's Plovers, St Helena Plovers and Madagascar Plovers, and White-fronted Plovers, as estimated in ARLEQUIN 3.5.2. Madagascar sampling sites were at Andavadoaka (Anda.), Asingo and Tsimanampetsose (Tsim.).

	Kittlitz's Plover				St Helena Plover	Madagascar Plover	White-fronted Plover		
	Kenya	Senegal	Anda.	Asingo	Tsim.	Tsim.	Namibia	Anda.	Tsim.
Kittlitz's Plover									
Kenya	–								
Senegal	0.034 <sup>†</sup>	–							
Anda.	0.027*	0.062*	–				0.432*	–	
Asingo	0.019	0.058 <sup>†</sup>	–0.012	–			0.495*	0.020 <sup>†</sup>	–
Tsim.	0.041*	0.083*	–0.006	–0.007	–				
St Helena Plover									
St Helena	0.414*	0.464*	0.424*	0.512*	0.441*				
Madagascar Plover									
Tsim.	0.277*	0.322*	0.247*	0.355*	0.297*	–			

\* $P < 0.001$ ; <sup>†</sup> $P < 0.01$ ; <sup>‡</sup> $P < 0.05$ .



**Figure 2.** (a–c) Genetic clustering among plover populations based on analyses of microsatellite data. Upper charts: principal component analysis (PCA); rectangles represent mean per cluster; ovals represent inertia ellipses per sampling site; eigenvalues are plotted for 50 principal components (PC 1 and PC 2 bars in black). Lower charts: STRUCTURE plots based on the number of genetic clusters ( $K$ ) with highest likelihood (see Fig. S1 for plots of additional  $K$ -values).



**Table 4.** Sequence divergence between island and mainland populations, among mainland populations, and between the Kittlitz's Plover (all populations) and its two island endemic sister species. Divergence (number of fixed differences in base pairs (bp)) between population pairs was assessed at two mitochondrial loci (*COI* and *ND3*) and four nuclear loci (*ADH5*, *MYO2*, *RAG1* and *FGB7*) using DNAsp. Where more than two mainland sites were analysed, and for species comparisons, the mean number of fixed differences between population pairs is presented. All 'island' populations were on Madagascar, with the exception of the St Helena Plover (see Fig. 1). S, number of polymorphic sites (bp). See Table S5 for more detailed sequence divergence values.

	Mitochondrial loci (1025 bp)			Nuclear loci (5299 bp)		
	S	Island–mainland divergence	Mainland–mainland divergence	S	Island–mainland divergence	Mainland–mainland divergence
Three-banded Plover	12	11.0 (1.1%)	–	7	2.0 (< 0.1%)	–
White-fronted Plover	17	9.0 (0.9%)	4.0 (0.4%)	16	12.0 (0.2%)	0.0 (0.0%) <sup>a</sup>
Kittlitz's Plover	2	0.3 (< 0.1%)	0.7 (0.1%)	51	8.0 (0.2%)	5.7 (0.1%)
Kittlitz's Plover – St Helena Plover	5	3.2 (0.3%)	–	52	6.4 (0.1%)	–
Kittlitz's Plover – Madagascar Plover	25	22.4 (2.2%)	–	58	15.0 (0.3%)	–
St Helena Plover – Madagascar Plover	22	21.0 (2.0%)	–	16	15.0 (0.3%)	–

<sup>a</sup>FGB7 sequence data not available.

endemic St Helena Plover and Madagascar Plover, the results of microsatellite analyses (based on allele size) were discordant with those of mitochondrial and nuclear sequence-based analyses. Whereas all populations of Kittlitz's Plovers appeared more similar to the Madagascar Plovers than did St Helena Plovers based on microsatellite analyses (STRUCTURE and PCA; interspecific pairwise  $F_{ST} = 0.300$  and  $0.451$ , respectively; Fig. 2c, Table 3), Kittlitz's Plover populations appeared more similar to St Helena Plovers than to Madagascar Plovers based on sequence data (Table 4).

### Inbreeding

Observed levels of heterozygosity ( $H_o$ ) were lower than expected ( $H_e$ ) in all study populations (Tables 5, S2 and S3), and inbreeding coefficients ( $F_{IS}$ ) across all sampled populations were positive (Table 5). However, inbreeding coefficients ( $F_{IS}$ ) among White-fronted Plover populations were not significant after correction for multiple testing (Table 5; adjusted nominal level for multiple comparisons:  $P = 0.001$ ) and among Kittlitz's Plover populations,  $P$ -values were significant at only two of the five sites (Andavadoaka in Madagascar and Kenya). Additionally, inbreeding coefficients for St Helena Plovers and Madagascar Plovers were non-significant (Table 5). All inbreeding coefficients remained qualitatively the same when assessed based on full sample sets, including individuals with relatedness of  $R > 0.5$ .

## DISCUSSION

### Intraspecific island–mainland genetic differentiation

Populations of three *Charadrius* species in Madagascar (White-fronted Plover, Kittlitz's Plover and Three-banded Plover) all demonstrated genetic divergence from their mainland African conspecifics. The level of divergence among Kittlitz's Plover populations was lower by an order of magnitude compared with White-fronted Plovers and Three-banded Plovers based on microsatellite loci (allele size) and mitochondrial sequence data. Additionally, Kittlitz's Plovers exhibited extremely low differentiation on the mainland and across larger distances (Kenya, Namibia, Senegal and South Africa; 6400 km apart) compared with White-fronted Plovers (Namibia and South Africa; 1200 km apart). The higher genetic connectivity of the Kittlitz's Plover compared with the White-fronted and Three-banded Plovers is consistent with differences in their behaviour and ecology (discussed in further detail below). Yet even for the particularly mobile Kittlitz's Plover, ocean barriers are an isolating factor, with clear genetic divergences from its two endemic island sister species, the Madagascar Plover and St Helena Plover.

White-fronted Plovers exhibited more pronounced genetic differentiation among island–mainland populations than did Kittlitz's Plovers. Given the high variation in genetic structure and phenotypic variation in plumage coloration

**Table 5.** Inbreeding coefficients ( $F_{IS}$ ) with associated  $P$ -values based on microsatellite data, as estimated in FSTAT 2.9.3, as well as mean expected heterozygosity ( $H_e$ ) and observed heterozygosity ( $H_o$ ), calculated based on the equation of Nei (1987), across markers for each sampling site (see Tables S2 and S3 for details of heterozygosity per marker). Madagascar sampling sites were at Andavadoaka (Anda.), Asingo and Tsimanampetsotse (Tsim.).

Species	Sampling location	$F_{IS}$ ( $P$ -value)	$H_e$	$H_o$
White-fronted Plover	Namibia	0.024 (0.291)	0.449	0.438
	Anda.	0.082 (0.037)	0.429	0.394
	Tsim.	0.102 (0.009)	0.351	0.316
Kittlitz's Plover	Kenya	0.120 (0.001 <sup>a</sup> )	0.775	0.684
	Senegal	0.112 (0.034)	0.775	0.693
	Anda.	0.202 (0.001 <sup>a</sup> )	0.726	0.587
	Asingo	0.086 (0.132)	0.748	0.691
	Tsim.	0.070 (0.177)	0.727	0.693
St Helena Plover	St Helena	0.222 (0.112)	0.167	0.131
Madagascar Plover	Tsim.	0.085 (0.128)	0.312	0.286

<sup>a</sup>Significant  $P$ -values after correction for multiple testing (adjusted nominal level for multiple comparisons:  $P = 0.001$ ).

observed across the White-fronted Plover species range (Hayman *et al.* 1986), we suggest that the current classification of island and mainland subspecies (as described by Delany *et al.* 2009) for the White-fronted Plovers of Namibia/South Africa and Madagascar (*Charadrius marginatus arenaceus* and *Charadrius marginatus tenellus*) may be appropriate. Similarly, we support the subspecies status of the Three-banded Plovers of Kenya and Madagascar (*Charadrius tricollaris tricollaris* and *Charadrius tricollaris bifrontatus*). Based on a limited sample size, we did not find sufficient support for the recent proposal of the latter as two distinct species (del Hoyo *et al.* 2014). We advise further genetic studies with larger sample sizes to clarify this, because for the *tricollaris* subspecies we were only able to include a single individual, sampled opportunistically in Kenya, in our study.

### Species differences in genetic structure

The differences identified in population-level divergence between Kittlitz's Plovers, White-fronted Plovers and Three-banded Plovers on Madagascar and mainland Africa may be due to several causative factors. Populations of Kittlitz's Plovers may exhibit greater genetic similarity due to more recent colonization of Madagascar, a larger

historical effective population size or higher levels of dispersal and population connectivity. Further evidence is required to determine colonization dates for these species, as well as historical population trends, although at present all three species number more than 10 000 mature individuals, across ranges of  $> 20\,000\text{ km}^2$  (Birdlife International, 2016c,d,e). Concerning dispersal, however, previous evidence does suggest that Kittlitz's Plovers have intrinsically higher levels of dispersal than the other two species (despite their overall physiological similarities) and that this may be linked to differences in habitat specialization and mating behaviour (Eberhart-Phillips *et al.* 2015, D'Urban Jackson *et al.* 2017).

Behaviour and ecology strongly influence the propensity of an organism to disperse away from their natal or breeding population (Greenwood & Harvey 1982, Phillimore *et al.* 2006), with consequences for population connectivity, gene flow and speciation (Edwards *et al.* 2005, D'Urban Jackson *et al.* 2017, Küpper & dos Remedios in press). Kittlitz's Plovers are generalist shorebirds found in areas of dried mud, short grass or sand. They are widely distributed across sub-Saharan Africa and are often opportunistic, adapting to novel environments that may not fit their optimal habitat requirements (Cassey 2002). In contrast, White-fronted Plovers are specialists with a preference for sandy areas and Three-banded Plovers favour firm sand, mud or gravel shores for breeding (del Hoyo *et al.* 1996). Kittlitz's Plovers also differ from the other study species in having a polygamous rather than monogamous mating system (Parra *et al.* 2014). Monogamous species often exhibit higher levels of philopatry and are more site-faithful, whereas in polygamous species, individuals may travel large distances to find multiple mates in different locations within and between breeding seasons (Reynolds & Cooke 1988, Cézilly *et al.* 2000, Pearson & Colwell 2013). Such dispersal is likely to increase gene flow between polygamous populations compared with monogamous ones (Eberhart-Phillips *et al.* 2015, D'Urban Jackson *et al.* 2017). In support of these dispersal differences, data on resightings of monitored individuals in Madagascar indicated greater distances of dispersal for the Kittlitz's Plover (resighted up to 113 km from capture site) than for the White-fronted Plover (resighted up to 15 km away; Zefania & Székely 2013, Eberhart-Phillips *et al.* 2015).

The St Helena Plover is currently the only endemic bird species on St Helena, and given the isolation of the island (almost 2000 km from the mainland), dispersal events here from the mainland are likely to be rare. It is therefore worth noting that successful colonization of this island has occurred only once, by ancestors of the Kittlitz's Plover, the most widely dispersing of the African endemic species. In contrast, Madagascar, a larger island closer to Africa, has been colonized more frequently and is currently home to four plover species (within *Clades a, e* and *f*; Fig. 1a). Long-distance dispersers may either have highly connected, genetically homogeneous populations (Belliere *et al.* 2000, Claramunt *et al.* 2012) or increased population divergence due to their propensity to colonize novel habitats (Shaw 1995, Ibrahim *et al.* 1996, Phillimore *et al.* 2006). Our results suggest that both cases hold true for the Kittlitz's Plover, with high connectivity across the mainland and stronger genetic differentiation leading to speciation following colonization of offshore islands.

### Species divergence on Madagascar and St Helena

Concerning the two endemic island species, the degree of genetic divergence of the Madagascar Plover and St Helena Plover from the widespread Kittlitz's Plover did not differ significantly from the observed intraspecific variation of the White-fronted Plover and Three-banded Plover. However, the endemic island species do exhibit strong phenotypic differences in both plumage coloration and body size, indicating that the genetic response to diversification may be lagging behind phenotypic traits (Adams *et al.* 2009, Avise 2009, Rheindt *et al.* 2011, Barth *et al.* 2013). Furthermore, when taken alongside the Kittlitz's Plover's extremely low intraspecific genetic structure, the observed genetic differences between the three species appear more prominent.

We predicted that the Kittlitz's Plover would be more genetically divergent from the St Helena Plover than from the Madagascar Plover given the smaller size and more isolated situation of the former population. However, there was discordance between microsatellite and DNA sequence data regarding these species' relationships. The Kittlitz's Plover appeared phylogenetically closer to the St Helena Plover based on mitochondrial and nuclear DNA sequences but more similar to the Madagascar

Plover at fast-evolving, microsatellite loci. Genetic discordance is not uncommon in phylogenetic studies and may occur due to incomplete lineage sorting, differences in selective pressures acting on different areas of the genome or adaptive introgression (Toews & Brelsford 2012). Errors in model estimation may also lead to genetic discordance, although in this case both sets of microsatellite analyses (STRUCTURE and PCA) were in agreement, and we evaluated sequence data directly (% sequence divergence). We therefore suggest an alternative theory, namely, that genetic drift is likely to be stronger on the smaller, more distant island of St Helena than on Madagascar, with greater impact on the faster-evolving microsatellite loci than on the slower-evolving nuclear and mitochondrial sequences. As such, microsatellite divergence between the Kittlitz's Plover and St Helena Plover is likely to be exaggerated and the signals of sequence data are more likely to reflect true phylogenetic history, with the Madagascar Plover being the more distant relative of the Kittlitz's Plover and St Helena Plover.

### Madagascar as a 'species pump'

Madagascar is sufficiently close to mainland Africa and large enough to offer a variety of habitats that many species have successfully colonized (Yoder & Nowak 2006). The identification of Madagascar as an important location in the genetic differentiation of the genus *Charadrius* is consistent with patterns reported for a wide range of flora and fauna, with the Mozambique Channel (approximately 400 km wide at its narrowest point) acting as a barrier to dispersal and gene flow between Madagascar and the mainland (Monaghan *et al.* 2005, Russell *et al.* 2008). In some taxa, the colonization of Madagascar has also been reported following dispersal across the Indian Ocean, with Madagascar acting as a stepping-stone between continents. For example, trans-oceanic dispersal between Madagascar, southern Asia and Australasia (as well as mainland Africa) is thought to have played a key role in the evolutionary radiation of multiple parrot genera (Schweizer *et al.* 2010).

For the *Charadrius* plovers, genetic divergence has followed colonization of Madagascar within each of the three African clades (Fig. 1a) and, for the Kittlitz's Plover 'superspecies' (*Clade e*), ancestral plovers have colonized Madagascar at least twice – the first time resulting in the emergence of the Madagascar Plover and the second time leading

to the presence of the modern Kittlitz's Plover population on the island. These results reflect patterns observed among other taxa, including the bat genus *Trienops* (Russell *et al.* 2008) and the small minnow mayflies (Ephemeroptera: Baetidae; Monaghan *et al.* 2005), of multiple dispersal events from mainland Africa towards Madagascar, resulting in the emergence of several independent lineages on this island. Our results confirm the importance of Madagascar as both a biodiversity hotspot and a location where new diversity may emerge. Thus, in the face of the current global biodiversity crisis (Woodruff 2001), our study further emphasizes the importance of conserving habitats that already foster numerous endemic species and genetically distinct populations, in the effort to preserve the future evolutionary potential of Earth's biota (Bowen 1999).

### Inbreeding among island populations

That all  $F_{IS}$  values were positive and all observed heterozygosity values were lower than expected across each sampled population (and for almost all markers assessed individually) would suggest some degree of inbreeding for all populations of small plovers studied here. Furthermore, as would be predicted for island endemics with small isolated populations, we found a particularly large proportion of highly related individuals ( $R > 0.5$ ) among the St Helena Plovers and Madagascar Plovers (65% and 42% of samples respectively, excluded from final analyses).

Nevertheless, contrary to our prediction, no strong heterozygote deficit emerged among study populations of St Helena Plovers, Madagascar Plovers, White-fronted Plovers or Kittlitz's Plovers according to microsatellite-based inbreeding coefficients ( $F_{IS}$ ), regardless of whether we included or excluded highly related individuals ( $R > 0.5$ ). Inbreeding may therefore not be a significant risk to population viability, even within the more isolated island population of the St Helena Plover. However, with population sizes only numbering several hundred individuals, these island endemics are nevertheless more vulnerable to extinction from adverse stochastic events or random catastrophes than are mainland plover populations that may number over several thousand, and where individuals have greater opportunities to disperse to more favourable locations (Lande 1993, Stork 2010, Warren *et al.* 2015).

## CONCLUSIONS

The emergence of genetic differentiation between Madagascar and mainland Africa, for populations of highly mobile African *Charadrius* plovers, highlights the importance of islands in driving genetic divergence and ultimately allopatric speciation. This role appears most significant for high dispersers such as the Kittlitz's Plover, where mainland populations appear highly connected and genetically homogeneous. Island colonization by ancestral Kittlitz's Plovers has led to both divergence of its modern-day Madagascar population and divergence of its two sister species, the endemic St Helena Plover and Madagascar Plover. This pattern mirrors that reported previously for the Kentish Plover, a widespread species with high genetic homogeneity across mainland Eurasia but clear genetic divergence across oceanic barriers, and multiple allopatric sister species (Küpper *et al.* 2012).

Despite the benefits of island colonization for driving the formation of species, island populations often suffer costs including low population sizes and increased risk of extinction. We therefore encourage conservationists to continue working towards the protection of island populations and their increasingly threatened habitats. Careful habitat management has already begun to improve prospects for the vulnerable St Helena Plover over the last decade, offering hope of reversing the declining trends that are common for isolated island populations.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** GenBank accession numbers for sequence data.

**Table S2.** Genetic variation and allele sizes for microsatellite loci assessed in the White-fronted Plover.

**Table S3.** Genetic variation and allele sizes for microsatellite loci assessed in the Kittlitz's Plover.

**Table S4.** Likelihoods of genetic clustering for all *K*-values assessed using STRUCTURE.

**Table S5.** Sequence divergence at mitochondrial and nuclear loci.

**Figure S1.** Plots of genetic clustering based on STRUCTURE for three *K*-values per dataset.