Conflict between Genetic and Phenotypic Differentiation: The Evolutionary History of a ‘Lost and Rediscovered’ Shorebird

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Citation

Published Version
doi:10.1371/journal.pone.0026995

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Conflict between Genetic and Phenotypic Differentiation: The Evolutionary History of a ‘Lost and Rediscovered’ Shorebird

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Abstract

Understanding and resolving conflicts between phenotypic and genetic differentiation is central to evolutionary research. While phenotypically monomorphic species may exhibit deep genetic divergences, some morphologically distinct taxa lack notable genetic differentiation. Here we conduct a molecular investigation of an enigmatic shorebird with a convoluted taxonomic history, the White-faced Plover (Charadrius alexandrinus dealbatus), widely regarded as a subspecies of the Kentish Plover (C. alexandrinus). Described as distinct in 1863, its name was consistently misapplied in subsequent decades until taxonomic clarification ensued in 2008. Using a recently proposed test of species delimitation, we reconfirm the phenotypic distinctness of dealbatus. We then compare three mitochondrial and seven nuclear DNA markers among 278 samples of dealbatus and alexandrinus from across their breeding range and four other closely related plovers. We fail to find any population genetic differentiation between dealbatus and alexandrinus, whereas the other species are deeply diverged at the study loci. Kentish Plovers join a small but growing list of species for which low levels of genetic differentiation are accompanied by the presence of strong phenotypic divergence, suggesting that diagnostic phenotypic characters may be encoded by few genes that are difficult to detect. Alternatively, gene expression differences may be crucial in producing different phenotypes whereas neutral differentiation may be lagging behind.


Received August 26, 2011; Accepted October 7, 2011; Published November 9, 2011

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Funding: Funding for fieldwork was provided by the National Geographic Society (8403-08). Genetic analyses were funded by the Natural Environmental Research Council (M09184), FER and CK were supported by postdoctoral fellowships of German Academic Exchange Service (DAAD). AK was supported by the Hungarian Scientific Research Fund (OTKA, K81953 to Ádám Miklósi). LW was supported by the National Natural Science Foundation of China-Guangdong Joint Fund (Grant No. U0833005). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Explaining the occurrence and maintenance of phenotypic variation has been a central theme in evolutionary biology for more than 150 years. Darwin [1] derived many of the insights and ideas for his seminal work by observing the phenotypic diversity of pigeon breeds. However, when present in natural populations, phenotypic variation still provides challenges for taxonomy and systematics today, and the molecular machinery behind it has only just begun to be unraveled.

Phenotypic characters have been the traditional taxonomic tool of choice and continue to contribute a major share to our current understanding of the Earth’s zoological diversity. Starting in the 1980s, however, a molecular revolution in the field of phylogenetics added DNA to the taxonomists’ toolkit [2]. Apart from corroborating most of our long-standing classification of animals, these new molecular data routinely refine our insights into relationships between groups for which phenotypic characters seem to have been harvested [3]. Occasionally, molecular results are at odds with previous phenotype-based hypotheses, which sometimes leads to a re-examination of the latter under more appropriate assumptions and an eventual removal of conflict [4].

Disagreement between phenotypic and molecular characters between closely related taxa can be due to inadequate data or false assumptions in at least one of the data sets, or it can be real and may point to fundamental underlying biological phenomena [5]. On the one hand, driven by molecular enquiries, there has been
Patterns of Differentiation in a Plover

Table 1. Morphometric, ecological, behavioral and plumage differences between *alexandrinus* and *dealbatus* as given by Kennerley et al. (2008) and their interpretation and score according to the criteria of Tobias et al. (2010).

<table>
<thead>
<tr>
<th>Character</th>
<th><em>alexandrinus</em></th>
<th><em>dealbatus</em></th>
<th>Interpretation of difference (as per Tobias et al. 2010)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plumage characters</td>
<td>dark lores in breeding plumage</td>
<td>white lores in breeding plumage</td>
<td>‘major’: different color of strongly demarcated body part</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>dull dark-brown upperparts</td>
<td>pale brown upperparts</td>
<td>‘medium’: different tone of significant area of feathering</td>
<td>2</td>
</tr>
<tr>
<td>Narrower black frontal bar on</td>
<td>narrower black frontal bar on forecrown of male breeding plumage</td>
<td>wider black frontal bar on forecrown of male breeding plumage</td>
<td>‘minor’: weak divergence in a plumage feature</td>
<td>1</td>
</tr>
<tr>
<td>Forecrown of male breeding</td>
<td>more dark on lower ear coverts</td>
<td>less dark on lower ear coverts</td>
<td>‘minor’, but potentially co-varying with previous traits</td>
<td>0</td>
</tr>
<tr>
<td>Plumage</td>
<td>more extensive dark patches on breast side</td>
<td>less extensive dark patches on breast side</td>
<td>‘minor’, but potentially co-varying with previous traits</td>
<td>0</td>
</tr>
<tr>
<td>Duller orange crown in</td>
<td>more vivid orange crown in breeding plumage</td>
<td>more vivid orange crown in breeding plumage</td>
<td>‘minor’, but potentially co-varying with previous traits</td>
<td>0</td>
</tr>
<tr>
<td>Breeding plumage</td>
<td>shorter wing</td>
<td>longer wing</td>
<td>effect size d = 0.448, i.e. ‘minor’</td>
<td>1</td>
</tr>
<tr>
<td>Biometric characters</td>
<td>shorter tarsus</td>
<td>longer tarsus</td>
<td>effect size d = 0.922, i.e. ‘minor’ (score 1), but co-varying with wing length</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>shorter bill</td>
<td>longer bill</td>
<td>effect size d = 0.340, i.e. ‘minor’ (score 1), but co-varying with wing length</td>
<td>0</td>
</tr>
<tr>
<td>Ecological and behavioral</td>
<td>on average inhabits softer mud along tidal channels</td>
<td>on average inhabits sandier substrate</td>
<td>‘minor’</td>
<td>1</td>
</tr>
<tr>
<td>characters</td>
<td>less active foraging behavior</td>
<td>more active foraging behavior</td>
<td>‘minor’ to trivial, but scoring limited to one trait</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Horizontal stance; head held into shoulders</td>
<td>Upright stance; neck visible</td>
<td>‘minor’, but scoring limited to one trait</td>
<td>0</td>
</tr>
<tr>
<td>Geographical relationship</td>
<td>Sympatric on migration and during winter; no information on contact of breeding</td>
<td></td>
<td></td>
<td>≥ 0</td>
</tr>
</tbody>
</table>

Biometric measurements were taken from table 1 in Kennerley et al. (2008). Note that various extremely minor traits are not listed as these would not have qualified for scoring. Also note that vocal differences are not given as none are known. For the score on geographical relationship, see Results. Final score amounts to 8.

doi:10.1371/journal.pone.0026995.t001
reconstruct the details of how its taxonomic identity became obliterated.

Documenting the true distribution and the pronounced phenotypic differences of a “lost” taxon of plover, Kennerley et al. [23] stopped short of elevating dealbatus to species level, instead calling for detailed molecular enquiries to examine the genetic distinctness of this enigmatic bird. Based on a large sampling regime of 278 individuals from across the globe (Fig. 2), we used three mitochondrial DNA (mtDNA) genes and seven microsatellites to investigate the evolutionary history of dealbatus. For a better understanding of the phylogenetic relationships of the Charadrius alexandrinus superspecies and to find the closest relative of dealbatus, we incorporated four other species that form part of the globally distributed C. alexandrinus complex (missing a fifth one, C. javanicus). Two of these (C. ruficapillus and C. peroni) as well as samples from five Asian populations of C. alexandrinus had not previously been available for phylogenetic research on the group [18]. To quantify phenotypic differences and further examine the biological species status of dealbatus, we also applied a recently proposed but promising phenotypic test of species delimitation [24,25] to biometric, ecological, behavioural and plumage data presented by Kennerley et al. [23]. Given the unusually distinct breeding-plumage colouration of this “lost and found” taxon, our goal was to establish whether dealbatus is indeed a member of the biological species C. alexandrinus, and, if so, whether it exhibits any population genetic differentiation.

Materials and Methods

Phenotypic species delimitation

We used the species delimitation criteria recently proposed by Tobias et al. [24] to assess the biological species status of dealbatus based on the morphometric, ecological, behavioral and plumage characters presented by Kennerley et al. [23] (Table 1). This species delimitation test assesses phenotypic differences between two taxa by assigning each character difference a score from 1 through 4, based on whether the difference can be considered ‘minor’, ‘medium’, ‘major’ or ‘exceptional’. If the sum of scores equals or exceeds a value of seven, the magnitude of the differences suggests the separation of taxa into two distinct biological species. For exact definitions of categories and assignment rules, see Tobias et al. [24].

Sampling regime and laboratory techniques

We obtained DNA from toe pad samples from museum specimens and blood samples from breeding and non-breeding populations of 278 plover individuals from across the globe (Fig. 2; Table S1 in File S1). DNA from toe pads was extracted using established protocols for museum samples at Swansea University [26]. DNA from blood samples was extracted as outlined by Kupper et al. [18].

For DNA samples extracted from blood we amplified three mtDNA genes: (1) a ~400-base-pair (bp) NADH dehydrogenase subunit 3 fragment (ND3, using the L10755 and H1151 primers from [27]); (2) a ~1200-bp sequence including partial fragments of the ATPase subunit 6/8 genes (ATPase 6/8, using the CO2GQL and CO3HMH primers from [28]), and (3) a ~700-bp partial D-loop fragment of the mitochondrial control region (CR, using TS778 and SNPL90 primers from [29,30]). All sequences are available at Genbank (accession numbers AM941499-AM941657, FR822997- FR823516, FR822850-FR822982, FR823147-FR823292), and the taxonomic identity and locality for each individual is listed in the Supplementary Material (Table S1 in File S1). For DNA obtained from toe pad samples that was partially degraded we designed primers to amplify shorter fragments. Details on these can be found in the Supplementary Material File S1. One sample from Saudi-Arabia (extracted from blood) was analysed using primers for both long and short fragments. The assembled sequences did not differ using the different primer sets and therefore we are confident that we amplified the same target region with primers for long and short fragment combinations.

MtDNA amplification conditions are given in Kupper et al. [18]. Initial PCRs for toe pad samples yielded little product and therefore we repeated the PCRs using 10 µg of product of the first PCR as a template. PCR products were sequenced at the NERC NBF at the University of Edinburgh. Sequences were edited using CODONCODE ALIGNER, version 3.4 (CodonCode, Dedham, Massachusetts). Only partial sequences with both forward and reverse strands available were used in subsequent analyses. To quantify sequencing error for mitochondrial DNA from six museum skin samples, were re-extracted and then blindly re-amplified and re-sequenced.

Fragment length differences in nuclear microsatellite markers were examined in 255 individuals from twelve plover populations using six autosomal microsatellite markers developed for the Kentish Plover and one marker developed for the barn swallow (Himantopus ruficollis) (Calex–05, –11, –14, –32, –35, –37, [31]; Hru2, [32]). Each sample was run in two multiplex PCRs (MRs) containing fluorescently labelled primers (MR 1: Calex–05, –35, and Hru2 primer set; MR 2: Calex–11, –14, –32 and –37). MRs with a total volume of 10 µL contained 2–8 µL mastermix solution (Qiagen, Valencia, California), ~2 µM of the primer mix, and 20 ng DNA. Relative primer concentrations were optimized to obtain similar peak sizes across different primer sets in the fragment analysis. MRs were performed in a thermal cycler (MJ Research model PTC DNA engine) according to the multiplex kit manufacturer’s default protocol: the program started with a 15-min activation cycle at 95°C followed by 35 cycles of 94°C for 30 s, annealing temperature (MR 1: 60°C; MR 2: 62°C) for 90 s, and 90 s at 72°C. The program finished with a 10-min extension cycle at 72°C. A fraction of the MR products was loaded onto the ABI 3730, and allele sizes were assigned using GENEMAPPER, version 3.7 (Applied Biosystems, Foster City, California). Some DNA samples of museum specimens were degraded and produced

Figure 1. Male breeding Kentish Plover (Charadrius alexandrinus alexandrinus left) and White-faced Plover (C. a. dealbatus right) at Tanjung Tokong (Penang, Malaysia) by D.N. Bakewell. Note the differences in facial coloration and tone of back plumage. doi:10.1371/journal.pone.0026995.g001
inconsistent genotypes with null and/or false alleles. Therefore, for all museum samples, microsatellite genotyping was repeated four times and we only used samples that i) produced consistent genotypes across the four runs and ii) for which genotypes from at least six of the seven markers could be retrieved for the subsequent analyses.

Phylogenetic methods

We first conducted phylogenetic analyses on individual mitochondrial loci. Subsequently, we concatenated individual loci, since all three genes are linked. The Akaike information criterion as implemented in the program jModelTest [33] was used to evaluate the best fit for each individual mtDNA gene (Table S2 in File S1). We employed maximum parsimony (MP) and Bayesian methods using the programs PAUP* 4.0b10 [34] and MRBAYES 3.1.2 [35], respectively. For details on analytical conditions of the PAUP and MRBAYES runs, see the Supplementary Material File S1.

We used a Shimodaira-Hasegawa test [36] by running 100 bootstrap replicates as implemented in PAUP to evaluate whether a tree topology constrained to alexandrinus and dealbatus monophyly had a significantly poorer fit to the sequence data than the phylogenetic tree topology obtained through Bayesian analysis. The concatenated dataset was used as input for this test, and the topology of the tree with the highest Bayesian posterior probability served as the model to compare the constrained topology against. Since separate evolutionary models cannot be specified for different data partitions of a concatenated dataset in PAUP*, the test was run three times, using each of the three evolutionary models that were found to have the best fit for each mtDNA gene, respectively.

Population genetic methods

Basic descriptive information on the microsatellites used can be found in Kupper et al. [18,31]. For microsatellite data consisting of seven loci in 180 individuals, the program ARLEQUIN version 3.11 [37] was used to compute FST values between samples unequivocally identified as dealbatus and alexandrinus. We employed the program STRUCTURAMA (http://fisher.berkeley.edu/structurama/index.html; [38]) to estimate the number of discrete populations (K) in our sampling regime. The program runs a Markov Chain Monte Carlo analysis under a Dirichlet process prior to approximate the posterior probability that individuals are assigned to specific populations. We ran two chains at 20,000,000 generations with a sampling frequency of 1,000 and excluded a burn-in of 10%.

We used STRUCTURE version 2.3.1 [39,40] to perform ten runs of the same microsatellite dataset at K = 1 and at K = 2. Ten independent simulations were run for 1,000,000 generations with a burn-in of 100,000 using the admixture model with correlated allele frequencies. Label switching among these ten runs was taken into account and they were combined using the program CLUMPP [41] with the “FullSearch” option enforced. Structure plots were then visualized using the program DISTRUCT [42].

Results

Phenotypic species delimitation test

Table 1 lists biometric, ecological, behavioural and plumage differences between alexandrinus and dealbatus as given by Kenmerley et al. [23], as well as the scores assigned to each character difference according to the phenotypic species delimitation test proposed by Tobias et al. [24]. The total score amounted to 8,
which is greater than the “species threshold” set by Tobias et al. [24] at 7, indicating that alexandrinus and dealbatus display a phenotypic differentiation that is typical for members of different species.

Sequencing and phylogenetic results

Our final alignment of overlapping CR sequences included 182 individuals and spans 380 bp, of which 116 bp were variable and 95 bp parsimony-informative. For ATPase 6/8, 168 individuals were successfully sequenced over 842 bp, of which 113 bp were variable and 90 bp parsimony-informative. Lastly, for ND3 we retrieved a 419 bp fragment for 183 individuals, 60 bp of which were variable and 45 bp parsimony-informative. The rate of sequence ambiguities for DNA samples from museum specimens was 0.3%. When concatenating our data, sequences for all three genes were available in 149 individuals spanning all the taxa examined.

Phylogenetic results for individual loci were highly congruent to the extent that no clade strongly supported by one gene was strongly contradicted by another (data not shown). The concatenated Bayesian analysis arrived at a phylogeny that corroborated the relationships uncovered by individual gene analyses and refined them with additional resolution (Fig. 3). MP analyses were usually less well-resolved than Bayesian analyses but were always in agreement.

The dealbatus mtDNAs were nested within a large and undifferentiated clade of alexandrinus mtDNAs from across Eurasia (Fig. 3), stretching from Spain in the west to Japan in the east. The combined clade of alexandrinus and dealbatus samples was characterized by high nodal support. Shimodaira-Hasegawa tests were carried out to compare maximum likelihood scores of the Bayesian concatenated topology (− ln L B) versus the score for a topology constrained to keep each dealbatus and alexandrinus monophyletic (− ln L C). Likelihood scores (− ln L B/− ln L C) were evaluated for the best-fit model of each locus (Table S2 in File S1), namely ATPase (5217.95/5628.45), ND3 (5137.72/5630.60) and CR (5204.57/5605.09). In each case, the difference between likelihoods of topologies was highly significant (p<0.005), indicating that there was no support for a tree in which dealbatus is monophyletic and sister to a monophyletic clade of alexandrinus samples.

We also recovered high nodal support for a monophyletic C. marginatus, although samples from mainland Africa and Madagascar are deeply diverged (Fig. 3). C. marginatus was placed as the sister species to C. peroni from south-east Asia with high support, with the clade consisting of these two species placed as the sister to C. alexandrinus (incl. dealbatus) with solid nodal support (Fig. 3). The closest relative of this lineage of three Old World plovers was the American C. nivosus, confirming previous results that C. nivosus is not the immediate sister of C. alexandrinus and must therefore be considered a species of its own [18]. Finally, within the limits of our sampling regime, C. ruficollis from Australia was the most basal plover species within the C. alexandrinus superspecies, with a high support for the monophyly of the other four species (C. alexandrinus, C. peroni, C. marginatus, C. nivosus).

Population genetic results

The FST value between alexandrinus and dealbatus was 0.012, indicating limited population genetic differentiation. In contrast, FST values between dealbatus and the three other species available for this analysis (C. ruficollis, C. marginatus, C. nivosus) ranged from 0.378 to 0.405, indicating a deeper level of genetic differentiation in agreement with the results of the mtDNA analyses. In addition, FST values among those three other species were pronounced (0.478 to 0.581).

Population structure analyses of the microsatellite data of 180 alexandrinus and dealbatus specimens (not including the other plover species) using the program STRUCTURAMA yielded a probability of 0.99 for K=1, i.e. that samples do not aggregate into multiple population clusters (with a residual probability of 0.01 for K=2). Despite the low probability for the presence of two distinct population clusters we used STRUCTURE to compute assignment probabilities for each individual in a 2-cluster scenario (Fig. 4). Even if the presence of two population clusters was assumed, each individual (including both dealbatus and alexandrinus samples from across the entire geographic distribution) possessed a roughly equal probability of being assigned to the first versus the second cluster, indicating that there was no detectable population-genetic differentiation either between alexandrinus and dealbatus, or across Eurasian sampling sites.

Discussion

Phenotypic versus genetic differentiation of an enigmatic shorebird

Kennerley et al. [23] documented the re-discovery of a forgotten taxon of shorebird, Charadrius alexandrinus dealbatus, based on a suite of biometric, ecological, behavioral and plumage characters that set this form apart from nominate alexandrinus. While stopping short of elevating dealbatus to species level, the authors considered it likely that the two taxa do not interbreed and constitute distinct biological species. Our application of a phenotypic species delimitation test confirms these findings and shows that the level of phenotypic differentiation between dealbatus and alexandrinus is on a par with that of known biological species pairs.

The phenotypic species threshold at 7 is based on a calibration using a large set of known avian sister species, but Tobias et al. [24] acknowledge that this threshold may not be uniformly applicable and may need to be fine-tuned or adjusted in certain taxonomic groups. Our score of 8 was above the species threshold, but is probably a minimum estimate, considering our tentative assignment of a zero score to the “geographical relationship” category (Table 1): depending on the extent to which two taxa’s geographic ranges come into contact, Tobias et al. [24] assign scores from 0 (for complete allopatry) through 3 (for parapatry), with no score assigned for sympatry since taxa automatically qualify for biological species status if they co-exist in sympatry. However, Tobias et al. [24] did not deal with migratory taxa such as alexandrinus and dealbatus, which are known to co-exist on migration or in the wintering grounds while keeping geographically separate on the breeding grounds. The level to which the breeding grounds of dealbatus and alexandrinus are separated is unknown: while it is unlikely that they breed sympatrically over a wide area, their breeding ranges may abut somewhere in Fujian Province (China) or they may form a narrow hybrid zone. Under these likely scenarios, a score of up to 5 could justifiably be added to the total, which would propel the test diagnostic to 11 and well beyond the gray zone around the threshold of 7. Note that vocal differences were not scored, as none are known. In summary, the application of the phenotypic species test corroborates conclusions that dealbatus and alexandrinus are phenotypically well-differentiated taxa probably worthy of species status [23].

The morphological diversification notwithstanding, dealbatus is characterized by little genetic differentiation from alexandrinus (Fig. 3, 4). In terms of mtDNA, they were virtually identical, and there were only weak differences in seven variable microsatellites.
Moreover mitochondrial trees constrained to keep all *dealbatus* samples in a monophyletic group and all *alexandrinus* samples in another are significantly less likely than the undifferentiated bush-like topology for these two taxa obtained through parsimony analysis. The near-identical genetic signature of *dealbatus* and *alexandrinus* can be explained by three non-exclusive reasons:

1. Our genetic markers may have been too crude or too few, failing to provide sufficient population-genetic resolution. If available in sufficient quantities, nuclear population-genetic markers can discriminate between recently-differentiated populations [43], so our seven microsatellites could be argued to be insufficient to detect differentiation between *alexandrinus*...
and dealbatus. However, we discount a potential lack of resolution for two reasons: (a) For the mtDNA, the Shimodaira-Hasegawa test was not expected to show a significantly poorer fit for the monophyly-constrained topology if the undifferentiated shallow topology of the alexandrinus-dealbatus clade (Fig. 3) were merely due to slow sorting. Instead, if dealbatus and alexandrinus had been on a different evolutionary trajectory for a long time, there should have been an underlying signal for their separation into monophyletic groups, even if the dataset were subject to varying levels of noise by incomplete sorting. (b) The application of the same three mtDNA genes uncovers considerable differentiation among closely related plovers (Fig. 3) and other birds. By the same token, our seven microsatellite markers are unlikely to be insufficient to detect differentiation at a taxonomically recognizable level, because they showed a deep differentiation in other plover species as highlighted by large FST values (>0.35) between closely related sibling species in the C. alexandrinus superspecies complex. While some of our microsatellites are found in conserved genomic regions, others are located in non-conserved ones [44]. Therefore, the FST values strongly suggest that our markers were sufficient.

(2) Even if they had reached species-level differentiation, dealbatus and alexandrinus may still occasionally exchange genetic material through introgression [5], especially considering that their breeding ranges may abut or narrowly overlap. Genetic introgression has been found in many birds (e.g. [45,46,47]) and mammals, including hominids [48]. However, while it is unknown how pervasive genetic introgression can be, present data on cases with genomic information indicate that introgression may only affect ~5% of the nuclear genome [48]. In our study, genetic markers came from both the nuclear and mtDNA genome, and our seven microsatellites were randomly distributed throughout the nuclear genome. Based on the genomic distribution of our markers, the simultaneous impact of introgression on all loci is unlikely.

(3) The low level of differentiation between dealbatus and alexandrinus may be genuine and extend to large parts of the genome. While more extensive nuclear locus sampling would be needed to rule out that we have not overlooked substantial differentiation based on marker choice, it is likely that dealbatus and alexandrinus join a list of unusual organisms that are characterized by great differentiation in phenotype but not in genotype, such as Darwin’s finches, Corvus corone crows, domesticated animals, certain lizards and our own species Homo sapiens (e.g. [9,10,11,12,13,14,49]).

The lack of differentiation in highly variable population genetic markers may indicate unimpeded gene flow between the two forms where their breeding ranges meet or overlap. Thus dealbatus may be at a phenotypically well-differentiated end of a plumage cline along the East Asian coastline, or there may be a relatively wide zone of intergradation between the two taxa in central eastern China. Studies of breeding populations to the north of Fujian Province are required to assess if there is any clinality or intermediacy of phenotypic traits. Based on the genetic evidence, we do not support calls for the elevation of dealbatus to biological species level. However, on account of phenotypic differences dealbatus should continue to be considered a subspecies of the Kentish Plover (C. alexandrinus).

**Potential mechanisms of the genetic-phenotypic disagreement**

Little is known about the underlying mechanisms that may account for a conflict between phenotypic and genotypic differentiation as seen in dealbatus and alexandrinus. It has been shown that speciation processes can be mediated by a select number of key genes, so called ‘genomic islands of speciation’, in the absence of any notable neutral genomic differentiation [50,51]. In birds, this has been confirmed in monarch flycatchers where radically different plumages are based on a single mutation [52].

Figure 4. Structure plot of 176 alexandrinus and dealbatus individuals for K = 2. Note that there was no difference in the assignment probabilities between dealbatus and alexandrinus samples. doi:10.1371/journal.pone.0026995.g004
On the other hand, new research into changes in gene expression (e.g. [53]) suggests that sequence data may contain limited information on the origin of important phenotypic differences between sister taxa. Key traits such as wing patches in flies [54] or beak size in Darwin’s finches [55] are confirmed to be subject to regulatory variation modulating gene expression. Similarly, Carrion and Hooded Crows (Corvus cornix cornix and C. cornix, respectively) are phenotypically well-differentiated (i.e. all black versus black-and-grey) but display limited genetic differentiation at 25 neutral nuclear introns [12]. However, there is notable differentiation in 1,300 genes expressed in the crows’ brains.

The most likely conclusion of our data is that the White-faced Plover is probably a young lineage whose phenotypic traits are encoded by a limited number of genes, whereas few additional genomic differences have so far accumulated. Its diagnostic plumage traits may additionally be governed by differences in gene expression that would be undetectable by sequence analysis. Future research on deathatus should (1) focus on candidate loci for plumage pigmentation (e.g. [52]) and (2) incorporate gene expression scans, since expression divergence may evolve faster than nucleotide divergence, possibly due to correlated effects that the change of expression of one gene has on other genes (e.g. [12]).

Phylogenetics of the C. alexandrinus superspecies

Our results provide the first glimpse into the evolutionary history of the Kentish Plover superspecies and establish the phylogenetic relationships of five members of this species complex with firm nodal support (Fig. 3). In particular, we confirm that C. nivosus and C. alexandrinus are not conspecific [18], and that south-east Asian C. peronii – which is not always considered a member of this superspecies (e.g. [19]) – is actually embedded in the complex and may constitute the sister lineage of African C. marginatus pending further sampling. Our data point to unusual levels of intra-specific differentiation between Malagasy and African populations of C. marginatus, but more data are needed to re-assess their level of differentiation.

Using both microsatellites and mtDNA genes, we sampled 208 individuals of C. a. alexandrinus (not including deathatus) from sites across the whole of Eurasia. We had a large sample size (n=15) for each Spain, Ukraine, Turkey, the Arabian peninsula, and Taiwan (including winter and summer individuals), and a moderate sample size (5≤n≤15) for the Indian subcontinent, Japan and north-east China, with additional samples from Xinjiang in central Asia (Fig. 2). C. alexandrinus displayed no detectable mtDNA (Fig. 3) or microsatellite (Fig. 4) differentiation across this vast range. This lack of differentiation supports previous findings that continental Eurasian populations of C. alexandrinus must be connected by high levels of gene flow [18]. However, more sampling of C. alexandrinus is required, especially in marginal and insular localities of its range (e.g. European or North African islands), to assess if this extreme genetic homogeneity extends to all populations.

In conclusion, we show that morphological and genetic differentiation are decoupled between White-faced and Kentish Plovers. In addition, our work reveals novel insights into the distribution of a cosmopolitan superspecies of shorebird that has served as a model organism in evolutionary and ecological research (e.g. [15,16,17]). To fully resolve the root of the conflicting morphological and genetic data, future research will benefit from the incorporation of genome-wide sequences, from a focus on candidate loci for plumage pigmentation or from gene expression scans to characterize expression divergence.

Supporting Information

File S1 Contains the following supporting material: Methods; Table S1. Sample identities and localities; Table S2. Evolutionary models and parameters selected for each locus by jModelTest.

Acknowledgments

We thank the Natural History Museum, Tring, UK for allowing us to sample toe pads under the loan number BS1300, BS1306 and BS1332. We also thank Dr. Anita Gamauf, bird curator at the Naturalhistories Museum at Vienna (Austria), for allowing us to sample toe pad specimens under her care. Jakob Augustin helped with lab work. The permit identity for the collection of C. ruficollis was DSE/PV permit 10004586. We thank two anonymous reviewers for helping improve the manuscript.

Author Contributions

Conceived and designed the experiments: FER CK TS. Performed the experiments: FER CK. Analyzed the data: FER CK. Contributed reagents/materials/analysis tools: TS PLML TB PRK DNB MA AK MAW T-L W-PL YS SJ SZ. CK. Wrote the paper: FER CK. Interpretation of the data: FER CK SVE TS.

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