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https://doi.org/10.1016/j.ympev.2015.04.010

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North or South? Phylogenetic and biogeographic origins of a globally distributed avian clade

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This is a preprint draft of an article published in Molecular Phylogenetics and Evolution 89: 151-159. doi: 10.1016/j.ympev.2015.04.010.
Abstract

Establishing phylogenetic relationships within a clade can help to infer ancestral origins and indicate how widespread species reached their current biogeographic distributions. The small plovers, genus *Charadrius*, are cosmopolitan shorebirds, distributed across all continents except Antarctica. Here we present a global, species-level molecular phylogeny of this group based on four nuclear (*ADH5*, *FIB7*, *MYO2* and *RAG1*) and two mitochondrial (*COI* and *ND3*) genes, and use the phylogeny to examine the biogeographic origin of the genus. A Bayesian multispecies coalescent approach identified two major clades (*CRD I* and *CRD II*) within the genus. Clade *CRD I* contains three species (*Thinornis novaeseelandiae*, *Thinornis rubricollis* and *Eudromias morinellus*), and *CRD II* one species (*Anarhynchus frontalis*), that were previously placed outside the *Charadrius* genus. In contrast to earlier work, ancestral area analyses using parsimony and Bayesian methods supported an origin of the *Charadrius* plovers in the Northern hemisphere. We propose that major radiations in this group were associated with shifts in the range of these ancestral plover species, leading to colonisation of the Southern hemisphere.

Keywords

*Charadrius*, phylogeny, nuclear genes, mitochondrial DNA, ancestral area analysis
1. Introduction

Molecular phylogenies can provide the means for estimating the geographic origins of widespread species and determining how they attained their current distribution (Avise, 2009; Edwards et al., 2012; Schweizer et al., 2011; Yu et al., 2014). However, establishing the exact phylogenetic relationships between species within a clade is often challenging. The history of phylogenetic inference for the Charadriiformes (shorebirds, gulls, alcids and allies) provides an example of such challenges. This order provides ideal study organisms for many areas of research since the taxa exhibit remarkable diversity in breeding systems, migratory behaviours, modes of offspring development, sexual size dimorphism, egg size and plumage colouration (Delany et al., 2009; Graul, 1973; Thomas et al., 2007; Piersma and Wiersma, 1996). Comparative analyses have made heavy use of existing phylogenies (Lislevand and Thomas, 2006; Székely et al., 2004a; Thomas et al., 2006a, 2006b) despite the relationships between many species not being fully resolved (Baker et al., 2012; Barth et al., 2013; Corl and Ellegren, 2013; Thomas et al., 2004).

Within the Charadriiformes, the genus Charadrius consists of 30 species of small plovers with highly diverse behavioural, ecological and life history traits, that breed on all continents except Antarctica. The phylogenetic history of the genus is controversial and, to date, molecular analyses have been based on only partially complete species datasets. The most complete molecular Charadrius phylogeny in terms of included taxa (26 species) was based on partial nuclear and mtDNA sequence data and outlined two major species clusters (Barth et al., 2013). Barth et al. (2013) controversially suggested the positioning of genera Vanellus, Phegornis, Anarhynchus, Thinornis and Elseyornis within the Charadrius clade, bringing into question the monophyly of the genus. This result was in contrast to traditional theories and phenotypic studies (Livezey, 2010), but supported earlier work based on allozymes and cyt b variation in a small number of species (four Charadrius species, Christian et al. 1992; 10 Charadrius species, Joseph et al., 1999). However, a limitation of the phylogeny presented by Barth et al. (2013) was the incomplete sampling of molecular markers (66% of sequences missing; 70% missing
characters). This can be problematic since phylogenetic analyses that rely on patchy datasets with large areas of missing data can lead to erroneous tree topologies (Lemmon, 2009; Roure et al., 2013) and therefore further analyses using more complete datasets are needed to more accurately establish the phylogenetic relationships within the genus.

The biogeographic origin of the *Charadrius* group is still debated. Contrasting Northern hemisphere and Southern hemisphere origins have been proposed. Proponents of the Northern hemisphere origin hypothesis have focussed on phenotypic characters, suggesting that the primitive *Charadrius* stock had breast bands, black lore lines and crown patches similar to the modern Palearctic-breeding common ringed plover (*C. hiaticula*; Bock, 1958; Graul, 1973). They speculated that these ornaments were reduced as their descendants colonised habitats with lighter coloured substrates. Additionally, Northern hemisphere proponents suggested that the ancestral species produced clutches of four eggs (as do *C. hiaticula* and neighbouring Palearctic species) from which species with reduced clutch sizes of two or three eggs evolved (Maclean, 1972). In contrast, in support of the Southern hemisphere origin hypothesis, similarities in the plumage patterns of the two-banded plover (*C. falklandicus*) of South America and the double-banded plover (*C. bicinctus*) of New Zealand were proposed as evidence of a close phylogenetic relationship, and the distribution of ten plover species at the southernmost tips of southern land masses were taken to be suggestive of a common ancestor inhabiting Antarctica at a time when the continent was not covered by ice (Vaughan, 1980). Support for a Southern Hemisphere origin was provided by an analysis of mitochondrial sequence variation in 15 plovers and allies (Joseph et al., 1999) that tentatively proposed South America as the ancestral home of this group. However, taxon sampling in this study was heavily biased towards species currently restricted to the Southern hemisphere whereas approximately half of the modern *Charadrius* species inhabit the Northern hemisphere (Hayman et al., 1986).

Here we attempt to more rigorously address the question of a Northern or Southern origin for the genus *Charadrius*. Recently-developed methods for ancestral area reconstruction include parsimony-based, likelihood-based and Bayesian models that statistically evaluate
alternative ancestral ranges at each node in a Bayesian phylogeny taking into account phylogenetic uncertainty (Heled and Drummond, 2010; Maddison and Maddison, 2015).

To achieve this goal we estimated the most comprehensive global molecular phylogeny of the *Charadrius* plovers to date, based on sequence data from two mitochondrial (mtDNA) and four nuclear loci and constructed using a Bayesian multi-species coalescent approach (*BEAST*; Drummond *et al.*, 2012; Heled and Drummond, 2010). With this phylogeny we investigated i) phylogenetic relationships within the genus *Charadrius* and ii) their biogeographic origins, using both parsimony-based and Bayesian methods (Maddison and Maddison, 2015; Yu *et al.*, 2014).

2. Material and methods

2.1. Taxon sampling

Samples were collected from three individuals for a total of 34 species (Table S1, Supplementary material) including 29 currently classified *Charadrius* species (all recognised species except *C. javanicus*) as well as five non-*Charadrius* species: four species with debated taxonomic classification, from closely-related genera (*Anarhynchus frontalis, Eudromias morinellus, Thinornis rubricollis, Thinornis novaeseelandiae*; Barth *et al.*, 2013; Bock, 1958; Davis, 1994; Nielsen, 1975; Vaughan, 1980) and one more distantly-related outgroup species, *Pluvialis squatarola*.

Blood samples were collected from 23 *Charadrius* species, and five non-*Charadrius* species from wild populations following methods outlined by Székely *et al.* (2008). Toe-pad samples were collected from museum specimens at the Natural History Museum, Tring, from six further *Charadrius* species (*C. alticola, C. asiaticus, C. forbesi, C. peronii, C. placidus* and *C. obscurus*).

2.2. DNA extraction, amplification and sequencing
DNA was extracted from blood samples using an ammonium acetate precipitation method (Nicholls et al., 2000; Richardson et al., 2001) at the University of Sheffield. To avoid cross-contamination with blood samples, DNA extraction from museum toe-pad skin samples was conducted in a separate, dedicated pre-PCR laboratory at a different location, Swansea University, using DNeasy Tissue Kits (Qiagen); see Bantock et al. (2008) for full protocol.

We amplified six loci using Polymerase Chain Reaction (PCR), two mtDNA loci: COI (cytochrome oxidase I) and ND3 (NADH dehydrogenase subunit 3), and four nuclear loci: ADH5 (alcohol dehydrogenase 5), FIB7 (β-fibrinogen intron 7), MYO2 (myosin-2/3) and RAG1 (recombination activating gene 1). These genes were selected based on their previous utility in species-level avian phylogenies (Chesser, 1999; Fain et al., 2007; Fain and Houde, 2007; Ericson et al., 2003; Hebert et al., 2004). For DNA extracted from blood samples, ‘universal’ avian primers were utilised (Table S2, Supplementary material). For DNA samples extracted from toe pads, primers targeting at least one shorter region per gene were used to handle degradation (Table S3, Supplementary material). For the mtDNA genes ND3 and COI, suitable primers were already available (Lee and Prys-Jones, 2008; Rheindt et al., 2011), including a set of three primer pairs designed to amplify the COI gene partially (‘D’, ‘L’ and ‘Q’ fragments; Table S3, Supplementary material). For nuclear genes, we designed new primers using Primer3 (Rozen and Skaletsky, 2000). New primers were located in conserved regions based on alignment of full Charadrius sequences to improve cross-species amplification (e.g. Küpper et al., 2008).

PCRs were conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler in 10 μl reaction mixes containing 4 μl Qiagen Multiplex Mix, 0.1 μM of each primer and 20-30 ng DNA. PCR conditions were as follows: 95°C for 15 min, followed by 42 cycles of 94°C for 30 s, Ts (primer specific annealing temperature, Tables S2 and S3, Supplementary material) for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. We ran a small aliquot of the PCR products on a 1% agarose gel to ensure amplification success. PCR products were then purified using 2 μl 10x diluted ExoSAP-IT (GE Healthcare) according to the instructions of the manufacturer and subsequently sequenced. Cycle sequencing was performed by GenePool Laboratory, Edinburgh,

### 2.3. Sequence alignment and phylogenetic analyses

Alignment of forward and reverse sequences, base-calling, end-clipping and ambiguity checks were carried out in CodonCode Aligner 3.7.1 (CodonCode Corporation) using the ClustalW algorithm for alignment. For nuclear genes, heterozygote positions were coded according to the universal ambiguity code. Full sequence alignments for each gene were produced in MEGA 5.21 (Tamura et al., 2011). Best-fit nucleotide substitution models were selected based on Akaike’s Information Criterion (AIC) in MrModelTest 2.3 (Nylander, 2004; Table 1). Sequence data has been deposited in the GenBank sequence database (accession numbers ADH5 GenBank: KM001088-KM001169; MYO2 GenBank: KM001170-KM001256; COI GenBank: KO001257-KM001341; ND3 GenBank: KM001342-KM001425; FIB7 GenBank: KM001426-KM001507; RAG1: KM001508-KM001594).

For eight cases, data was retrieved from GenBank or the Barcode of Life Database (BOLD). These sequences included: *C. hiaticula* COI (GenBank: GU571812.1, GU571811.1, GU571331.1; Johnsen et al. unpubl.), *C. falklandicus* COI (GenBank: FJ027346.1, FJ027345.1; Kerr et al. 2009), *C. leschenaultii* COI (GenBank: DQ432845.1, GQ481569.1; Kerr et al., 2007, 2009), *C. mongolus* COI (GenBank: GQ481572.1, GQ481571.1, GQ481570.1; Kerr et al., 2009), *C. ruficapillus* ND3 (GenBank: FR823187.1, FR823188.1, FR823189.1; Rheindt et al., 2011), *A. frontalis* COI (BOLD: BROM379-06, BROM380-06, BROM617-07; A.J. Baker), *E. morinellus* COI (GenBank: DQ433492.1, GU571813.1, GU571814.1; Kerr et al., 2007; Johnsen et al. unpubl.) and *E. morinellus* RAG1 (GenBank: EF373182.1; Baker et al., 2007).

Phylogenetic analyses were performed in *BEAST 1.7.5* (Drummond et al., 2012; Heled and Drummond, 2010) using XSEDE on the CIPRES (Cyberinfrastructure for Phylogenetic Research) gateway (Miller et al., 2010). *BEAST employs a Bayesian multispecies coalescent approach and is capable of estimating divergence times, rates of gene evolution and the
parameters of evolutionary models for separate gene partitions. This program co-estimates multiple gene trees embedded in a shared species tree, allowing for variation in rates of molecular evolution between loci.

Xml-files for *BEAST analyses were prepared using BEAUti 1.7.5 (BEAST package) with all sequences concatenated and each gene assigned to a separate substitution model partition and clock model partition. Since the number of informative sites was often low for individual genes (i.e. high mean pairwise % identity, see Table 1), the data were grouped into two partition trees, one for mitochondrial COI and ND3 genes and one for the five nuclear genes (ADH5, FIB7, MYO2, ND3 and RAG1). The species tree prior was set to Yule Process and the population size model set to piecewise linear and constant root. Mean substitution rates under an uncorrelated log-normal relaxed clock (ucld means) were estimated based on a uniform distribution prior with range 0 - 10 and an initial value of 1 (Ferreira and Suchard, 2008). The Markov chains were run for 400 million generations and sampled every 15,000 generations.

Convergence was concluded from stationary distributions of MCMC (Markov chain Monte Carlo) sample traces in Tracer v1.5 (BEAST package). Summary of the posterior distribution of *BEAST trees and identification of the maximum clade credibility (MCC) tree was conducted using TreeAnnotator 1.7.5 (Drummond and Rambaut, 2007) with a burn-in value of 15% and median node heights. Three independent runs were conducted for each treatment to ensure convergence. The MCC tree was visualised in FigTree v1.4.0 (available at: http://tree.bio.ed.ac.uk/software/figtree/).

Nucleotide composition and transition/ transversion ratios for each gene region were calculated in MEGA 5.21 (Tamura et al., 2011). Compositional stationarity was assessed based on disparity index values (MEGA 5.21) and chi-squared tests of heterogeneity (PAUP* 4.0a142; Swofford, 2002). Saturation plots were generated using DAMBE 5.5.29 (Xia, 2013). In order to compare phylogenetic estimates for each of the six gene regions, we conducted an additional analysis in *BEAST, as above, but with each gene assigned to a separate, unlinked partition tree.
Pairwise topological similarity between gene regions was evaluated based on maximum clade credibility trees for each gene partition, using Compare2Trees (Nye et al., 2006).

2.4. Ancestral area reconstruction

The present-day breeding distributions of each species (data from International Union for Conservation of Nature (IUCN), 2014) were categorised into nine geographic regions. These regions were based on terrestrial zoogeographic realms and modified in line with data on phylogenetic turnover among regions in birds (Holt et al., 2013) as well as the distribution of *Charadrius* plovers, such that each region is occupied by at least four modern *Charadrius* species (Fig. 1; Table S1, Supplementary material).

For reconstruction of ancestral areas, we employed two methods that statistically evaluated alternative ancestral ranges at each node of the summarised Bayesian phylogeny, taking into account phylogenetic uncertainty. The first was a parsimony method implemented in Mesquite 3.02 (Maddison and Maddison, 2015; Ancestral States Reconstruction Package) with a step (cost) matrix model. This model accounts for the likelihood of dispersal between regions. We assumed equal transition costs for movement between adjacent realms, therefore the costs reflected the minimum number of neighbouring areas a species would have to disperse through to make the transition.

Secondly, Bayesian binary Markov chain Monte Carlo (BBM) analysis was implemented in RASP v2.1 beta (Reconstruct Ancestral States in Phylogenies; Yu et al., 2010; Yu et al., 2014) under a variable F81+G model for 5,000,000 generations with 10 chains sampling every 100 generations and outgroup root distribution.

3. Results

3.1. Sequence characteristics
Properties of sequence data for each of the four nuclear and two mtDNA loci, including sequence length, nucleotide substitution models, percentage of variable positions, nucleotide composition and transition/transversion ratios are given in Table 1. The full sequence length of all six concatenated genes was 4295 base pairs. For DNA extracted from museum toepads the length of sequence data amplified was 1343 base pairs leading to 69% missing data in six species. No sequence data could be generated or retrieved for the following species/DNA fragments: C. bicinctus/COI, C. placidus/ND3 and RAG1, C. melodus/MYO2 and ND3, C. ruficapillus/ADH5 and E. morinellus/ND3, ADH5, FIB7 and MYO2. A total of 85% of characters were available across species and loci.

3.2. Phylogeny

The MCC tree supported the division of the genus Charadrius into two major clades (CRD I and CRD II; Fig. 1b). We have further categorised these two major clades into minor clades of sister species (Clades a – f; Fig. 1b) with largely shared geographic distributions and/or morphological characteristics (Bock, 1958; IUCN 2014; Nielsen, 1975). Of the taxa currently classified outside the genus, three species emerged within the CRD I clade (T. novaeseelandiae, T. rubricollis and E. morinellus), and one species within CRD II (A. frontalis).

Concerning phylogenetic relationships within the genus (Fig. 1b), Charadrius Clade CRD I included the ringed plover species, identified as minor Clade a (such as the common ringed plover, C. hiaticula and little ringed plover, C. dubius), a group which also included two species not presently classified as members of the genus Charadrius: T. rubricollis and T. novaeseelandiae. Additionally, CRD I included two species morphologically distinct from the ringed plover group in terms of body size and plumage colouration, namely E. morinellus and C. modestus. These two species were basally positioned in the CRD I lineage.

Within CRD II, we outlined five minor clades: Clade b included four Asian red-breasted species; Clade c included three Oceanian species - C. bicinctus, C. obscurus and one currently non-Charadrius species, A. frontalis; Clade d consisted of five American (Nearctic and
Neotropical) species of the mountain and plains plover group (Vaughan, 1980); Clade e included three African species - two endangered island species (C. thoracicus and C. sanctaeheleena) and one widespread species (C. pecuarius); and Clade f consisted of six species of the sand plover group, including C. alexandrinus and its allopatric sister species.

For the six species for which museum toepad samples were the source of DNA (C. alticola, C. asiaticus, C. forbesi, C. peronii, C. placidos and C. obscurus), sequence information was incomplete. Additionally, four Charadrius species were missing sequence data for one or two genes, namely C. bicinctus, C. placidos, C. melodus and C. ruficapillus, and only two gene regions were included for E. morinellus. Nonetheless, the phylogenetic placement of these species with partial or missing data did not differ from expectations based on geographical distribution or plumage colouration (Nielsen, 1975; Hayman et al., 1986).

Levels of nodal support, based on posterior probability, were above 0.9 for 20 of the 33 nodes and overall assignment of sister species to the six minor clades was highly supported (Fig. 1b). Nine nodes across the MCC tree had posterior probabilities below 0.7 (Fig. 1b). Five of these nodes were within Clade a and two within Clade d, where some species-level relationships could not be resolved with certainty. The final two nodes with notably low support (posterior probability <0.7) emerged at the basal node adjoining Clades e and f, and at the node linking the CRD I and CRD II clades. These nodes are likely to indicate areas of discordance between genes as pairwise topological similarity scores between gene regions ranged from only 72.1 to 81.2% (Table 2). Individual MCC gene trees, were often unable to resolve nodes with high support, though differences were restricted to species relationships in recently diverged clades (Fig. 2). Concordance between the two grouped tree partitions (mitochondrial and nuclear) was high with no discordance in species placement between clades (Fig. S1, Supplementary material).

### 3.3 Sequence stationarity and substitution saturation

No significant compositional heterogeneity was observed based on mean disparity index values or chi-squared tests (see Table 3) suggesting stationarity across sequences. Levels of
substitution saturation at third codon positions differed between genes. Saturation plots for
ADH5, MYO2 and RAG1 indicated near-linear divergence of transitions and transversions over
genetic distance (Fig. S2, Supplementary material). However, for the two mitochondrial genes,
ND3 and COI, non-linear trends emerged for both transitions and transversions, suggesting
saturation at F84 distances above 1.2 (COI) or 0.7 (ND3). For FIB7 (intronic DNA), transitional
saturation was reached by an F84 distance of 0.4. At this locus, ingroup Charadrius data
corresponded to F84 distances of <0.11, whereas distances of 0.38 – 0.59 appeared between
Charadrius spp. and Pluvialis squatarola suggesting saturation issues at the inter-genus level.

Differences in substitution saturation appeared associated with increased discrepancies
in species placement in individual gene trees relative to the MCC species tree. On average, six
species differed in clade positioning where saturation issues were present (COI, ND3 and FIB7)
compared to four species for other loci (Fig. 2).

3.4. Ancestral area reconstruction

Based on parsimony analysis, the most recent common ancestor of the Charadrius plovers (CRD
I and CRD II) originated in the Northern hemisphere, with a distribution in the Arctic and/or
Palearctic regions (Fig. 1). The BBM analysis concurred in identifying the Northern hemisphere
as the centre of origin but identified the Nearctic and Arctic regions as the most likely ancestral
range. The probability that the genus originated in the Southern hemisphere was <0.01
(parsimony) and <0.05 (BBM). Similarly, both major clades CRD I and CRD II emerged in the
Northern hemisphere based on both parsimony and BBM analyses. The most recent common
ancestor (MRCA) of clade CRD I was likely to have occupied a similar Northern hemisphere
range to the Charadrius MRCA, whereas the CRD II clade was more likely to have emerged in
Central Asia or the Palearctic than in Arctic or Nearctic regions (Fig. 1). BBM analysis confirmed
the Northern hemisphere origin of CRD II (Southern hemisphere probability < 0.14), though this
method did not provide strong resolution, instead identifying five regions with similar
probabilities for the MRCA of the clade.
During evolutionary radiation from their Northern origins, ancestral *Charadrius* plovers appear to have moved southwards and colonised Africa three times (within Clades a, e and f) and the Oriental - Oceanian regions at least three times (within Clades a, c and f). Consistently, the two extant species (*C. alticola* and *C. falklandicus*) that breed solely within the Neotropical region diverged within *Clade d* from North American ancestors.

4. Discussion

4.1 Phylogenetics and taxonomy

Our global molecular phylogeny of the *Charadrius* plovers evaluates the controversial evolutionary history of the genus based on the most extensive and complete molecular dataset to date. Our results supported the presence of two major clades (*CRD I* and *CRD II*) within the genus, and also supported the recent classification of the wrybill (*A. frontalis*) and the two *Thinornis* species, *T. rubricollis* and *T. novaeseelandiae*, as members of the genus as suggested by Barth *et al.* (2013). Furthermore, we include *C. modestus* and *E. morinellus* within *CRD I*, but note that these species appear to have diverged from other *CRD I* species early in the history of the clade.

Our results were based on a Bayesian multi-species coalescent approach, including 85% complete sequence characters from four nuclear and two mtDNA loci, and including all but one *Charadrius* species (*C. javanicus*), in order to recover the most likely species tree, taking into account levels of phylogenetic uncertainty and discordance across gene trees (Brito and Edwards, 2009; Corl and Ellegren, 2013). We identified six minor clades of sister species within the genus and these were strongly coherent with geographic distributions and morphological characters (e.g. plumage colouration; Bock, 1958; IUCN 2014; Nielsen, 1975). Of particular conservation interest is the result that the Kittlitz’s plover (*C. pecuarius*) of Africa was the closest widespread sister species of the ‘critically endangered’ St Helena plover (*C. sanctaehelenae*) and ‘vulnerable’ Madagascar plover (*C. thoracicus*).
Whilst our results largely supported those of Barth et al.’s (2013) partial dataset, there was disagreement in two areas. Firstly, we provided new evidence on the positioning of one Nearctic-breeding species, *C. montanus*, as a sister species to the Nearctic and Neotropical species *C. collaris*, *C. wilsonia*, *C. alticola* and *C. falklandicus* (*Clade d*), whereas Barth et al. (2013) suggested that *C. wilsonia* shared more recent ancestry with the Afrotropical *C. pecuarius* and Oceanian *C. ruficapillus* (*Clades e and f* respectively). Secondly, disagreement emerged concerning the order of divergence among *Clades d, e* and *f* within *CRD II*. Additionally, our analyses included three species (*C. tricollaris*, *C. forbesi* and *C. placidus*) as members of *Clade a*, within *CRD I*, that were not studied by Barth et al. (2013).

Whilst the members of *Clade a* were confidently assigned to this group (posterior probability 0.96), our analyses could not elucidate finer scale species-level relationships here with high probability. This was also the case for *Clade d*. We suggest that these areas of uncertainty emerged due to discordance in the phylogenetic signals of the six gene regions analysed since topological similarity between gene trees was only 72.1 to 81.2% and differences occurred in nucleotide composition and base substitution saturation. In particular, we observed signs of substitution saturation in mtDNA (*COI* and *ND3*) and *FIB7* regions.

Already identified as a major issue in recovering phylogenetic histories at deeper levels of the Charadriiform tree (Baker et al., 2012; Corl and Ellegren, 2013), discordance between gene trees is common when lineages have emerged following periods of rapid radiation (Jarvis et al., 2014). Such radiation commonly leads to incomplete lineage sorting, making it difficult to disentangle true orders of divergence in species trees (Chung and Ané, 2011; Corl and Ellegren, 2013; Degnan and Rosenberg, 2006; Jarvis et al., 2014; Knowles and Chan, 2008). Speciation is often a gradual, extended process rather than a single point event and gene flow commonly occurs after initial divergence (Avise and Walker, 1998). Additionally, hybridisation or introgression between species can enable gene flow even after species divergence (Kubatko, 2009).
Recent statistical advances in coalescent-based methods have indicated that statistically grouping genes into subsets can improve the accuracy of species tree estimation (Mirarab et al. 2014). Our analyses also benefited from this approach. Initial *BEAST MCMC models failed to converge with each gene assigned to separate gene tree partitions, suggesting that the number of informative sites per gene was often low. To optimise the analyses, we therefore grouped the six gene regions into two partition trees (mitochondrial and nuclear), whilst allowing estimation of separate substitution models and clock models for each region.

Technical advances in sequencing methodology and further reduction of sequencing and assembly costs will soon help to determine with greater confidence the evolutionary relationships between all species. These advances, facilitating multiple-locus and genome-wide sequence analyses, will enable the use of a newly emerging phylogenomic approach to infer evolutionary history (Delsuc et al., 2005, Jarvis et al., 2014) and should provide greater resolution in uncertain areas of the Charadrius phylogeny in future.

4.2. Biogeographic origin

Our results strongly supported a Northern hemisphere rather than Southern hemisphere origin for the genus Charadrius. Parsimony and BBM analyses were consistent in their support for the Northern hemispheric origin. The modern CRD I species are largely distributed within the Northern hemisphere (seven Northern, four Southern) whereas CRD II species breed largely in the Southern hemisphere (eight Northern, 13 Southern). Despite their differing distributions both major clades were identified as originating in the Northern hemisphere based on parsimony analysis (CRD I Palearctic/Arctic, CRD II Palearctic/ Central Asian origins). The BBM results included high uncertainty over the biogeographic region of origin of Charadrius. However, unlike the parsimony analysis, BBM models do not take into account geographical connectivity and likelihood of dispersal between regions (Yu et al., 2014). Nevertheless, the four biogeographic regions with highest support according to BBM for the MRCA of CRD II (probabilities 0.19 – 0.29) and the top two regions for the MRCA of CRD I
(probabilities 0.27 and 0.50) were located in the Northern hemisphere. Based on these results we suggest that the MRCA of the genus *Charadrius* was distributed in the Northern hemisphere, and that southwards dispersal led to subsequent colonisation of the Southern hemisphere. These Northern hemisphere origins match large-scale biogeographic patterns reported across a range of avian and mammalian taxa (Hunt, 2004; Maguire and Stigall, 2008; Schweizer *et al.*, 2011; Zachos *et al.*, 2001) and may have been important in driving speciation within the genus.

**5. Conclusions**

Our new phylogeny of the genus *Charadrius* provides much needed information on the evolutionary history of a diverse group of shorebirds. This group is emerging as an ideal model for studying the evolution of a range of phenotypic traits including breeding systems, migration strategies and plumage (Argüelles-Ticó, 2011; Owens *et al.*, 1995; van de Kam *et al.*, 2004), yet to date, studies on the genus *Charadrius* have only focused on microevolutionary patterns, investigating just one or a few closely related species (e.g. Székely *et al.*, 2004b; Vincze *et al.*, 2013). This updated phylogeny which largely shows agreement of molecular and phenotypic characters in the genus *Charadrius* provides a more robust framework to enable larger scale investigations into macroevolutionary changes within the clade. Whilst resolving many areas of taxonomic controversy, our phylogeny also highlights key points of uncertainty. Future phylogenetic studies should aim to resolve these points by examining more molecular markers, and by making use of advancements in sequencing technologies.

**Acknowledgements**

Samples were generously provided by Bernard Amakobe, Matilde Alfaro, Araceli Argüelles-Ticó, Marcela Beltrán, Fiona Burns, Stephen J. Dinsmore, Jim de Fouw, Balazs Kaholek, Sylvester Karimi, Terje Lislevand, Gabriel García-Peña, Susan Haig, Craig Millar, Christine Ochieng, Ian Owens, Jorge Parra, Paul D. B. Skrade, James St Clair, Bela Tokody, Pavel Tomkovich, Mike
Weston and Sama Zefania. We also thank Mark Adams, Robert Prys-Jones and Douglas Russell for sample collection from museum specimens at the Natural History Museum, Tring. Sequence analysis was supported by NERC-Biomolecular Analysis Facility at the University of Edinburgh and at the University of Sheffield. Gavin Thomas provided statistical advice on phylogenetic analyses and Allan Baker kindly commented an earlier draft of this manuscript. This study was funded by the Natural Environment Research Council as part of the PhD research of NdR (studentship no. NE/H525011/11 to TS). CK was supported by a Marie Curie IEF postdoctoral fellowship. Permits for fieldwork and collection of samples were provided by the national agencies in each country.

References


Yu, Y., Harris, A.J. & He, X. 2014. RASP (Reconstruct Ancestral State in Phylogenies) 3.0. Available at: http://mnh.scu.edu.cn/soft/blog/RASP.

Table 1: Characteristics of mitochondrial and nuclear loci used in the phylogenetic analysis of *Charadrius* and seven allied species.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>Nucleotide frequencies (%)</th>
<th>Transition/transversion bias (R)</th>
<th>Mean pairwise identity (%)</th>
<th>Nucleotide substitution model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh samples</td>
<td>Museum samples (% of full sequence)</td>
<td>AT</td>
<td>CG</td>
<td></td>
</tr>
<tr>
<td>ND3a</td>
<td>401</td>
<td>200 (50)</td>
<td>51.3</td>
<td>48.7</td>
<td>2.7</td>
</tr>
<tr>
<td>COIb</td>
<td>626</td>
<td>429 (69)</td>
<td>57.0</td>
<td>43.0</td>
<td>3.9</td>
</tr>
<tr>
<td>ADH5b</td>
<td>829</td>
<td>150 (18)</td>
<td>54.5</td>
<td>45.5</td>
<td>4.6</td>
</tr>
<tr>
<td>FIB7b</td>
<td>840</td>
<td>138 (16)</td>
<td>63.4</td>
<td>36.6</td>
<td>1.4</td>
</tr>
<tr>
<td>MYO2b</td>
<td>688</td>
<td>209 (30)</td>
<td>52.3</td>
<td>47.7</td>
<td>2.5</td>
</tr>
<tr>
<td>RAG1b</td>
<td>911</td>
<td>217 (24)</td>
<td>53.4</td>
<td>46.6</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*a* mitochondrial loci; *b* nuclear loci

Table 2: Pairwise topological scores (% similarity) between maximum clade credibility (MCC) trees constructed for each gene region.

<table>
<thead>
<tr>
<th></th>
<th>COI</th>
<th>ND3</th>
<th>ADH5</th>
<th>FIB7</th>
<th>MYO2</th>
<th>RAG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND3a</td>
<td>-</td>
<td>81.2</td>
<td>74.1</td>
<td>73.2</td>
<td>76.0</td>
<td>80.3</td>
</tr>
<tr>
<td>COIb</td>
<td>81.2</td>
<td>-</td>
<td>73.8</td>
<td>76.7</td>
<td>80.2</td>
<td>76.2</td>
</tr>
<tr>
<td>ADH5b</td>
<td>74.1</td>
<td>73.8</td>
<td>-</td>
<td>73.9</td>
<td>75.2</td>
<td>76.2</td>
</tr>
<tr>
<td>FIB7b</td>
<td>73.2</td>
<td>76.7</td>
<td>73.9</td>
<td>-</td>
<td>72.1</td>
<td>73.8</td>
</tr>
<tr>
<td>MYO2b</td>
<td>76.0</td>
<td>80.2</td>
<td>75.2</td>
<td>72.1</td>
<td>-</td>
<td>75.8</td>
</tr>
<tr>
<td>RAG1b</td>
<td>80.3</td>
<td>76.2</td>
<td>76.2</td>
<td>73.8</td>
<td>75.8</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* mitochondrial loci; *b* nuclear loci

Table 3: Mean disparity index values (I$_D$) and chi-squared tests of compositional heterogeneity for each gene region and codon position.

<table>
<thead>
<tr>
<th>Gene</th>
<th>All</th>
<th>1$^{st}$ codon</th>
<th>2$^{nd}$ codon</th>
<th>3$^{rd}$ codon</th>
<th>1$^{st}$ codon</th>
<th>2$^{nd}$ codon</th>
<th>3$^{rd}$ codon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>l$_0$</td>
<td>$\chi^2$</td>
<td>p</td>
<td>l$_0$</td>
<td>$\chi^2$</td>
<td>p</td>
<td>l$_0$</td>
</tr>
<tr>
<td>COIa</td>
<td>0.012</td>
<td>24.87</td>
<td>&gt;0.99</td>
<td>0.034</td>
<td>13.79</td>
<td>&gt;0.99</td>
<td>0.011</td>
</tr>
<tr>
<td>ND3a</td>
<td>0.026</td>
<td>13.8</td>
<td>&gt;0.99</td>
<td>0.075</td>
<td>31.57</td>
<td>&gt;0.99</td>
<td>0.006</td>
</tr>
<tr>
<td>ADH5b</td>
<td>0.006</td>
<td>34.8</td>
<td>&gt;0.99</td>
<td>0.013</td>
<td>23</td>
<td>&gt;0.99</td>
<td>0.01</td>
</tr>
<tr>
<td>FIB7b</td>
<td>0.006</td>
<td>24.3</td>
<td>&gt;0.99</td>
<td>0.001</td>
<td>17.59</td>
<td>&gt;0.99</td>
<td>0.01</td>
</tr>
<tr>
<td>MYO2b</td>
<td>0.00</td>
<td>80.16</td>
<td>0.88</td>
<td>0.0</td>
<td>37.11</td>
<td>&gt;0.99</td>
<td>0.0</td>
</tr>
<tr>
<td>RAG1b</td>
<td>0.004</td>
<td>22.39</td>
<td>&gt;0.99</td>
<td>0.0</td>
<td>9.41</td>
<td>&gt;0.99</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*a* mitochondrial loci; *b* nuclear loci
Figure 1: a) Biogeographic regions (revised from Holt et al., 2013) used to define current breeding distributions for each species. b) The maximum clade credibility tree for 29 *Charadrius* and five species currently assigned to different genera. Results of parsimony ancestral area analysis are shown for all nodes (pie chart colours by region) and Bayesian binary Markov chain Monte Carlo (BBM) results with probability distributions are added for the three basal *Charadrius* nodes (larger pie charts). Minor clades within CRD I and CRD II are labelled a – f. Posterior probabilities are indicated at each node.
Figure 2: *BEAST maximum clade credibility trees based on sequence data from six genes: a) COI, b) ND3, c) ADH5, d) FIB7, e) MYO2 and f) RAG1. Mitochondrial gene. Branches are shaded according to posterior probability. Black species labels indicate matches to MCC species tree (Fig. 1); grey and red labels indicate differences to MCC species tree placement, either within or between minor clades respectively.