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

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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 *Charadriu*s 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, T_a (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,

on an ABI 3730 DNA analyser (Applied Biosystems) using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

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. sanctaehelenae*) 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. placidus* and *C. obscurus*), sequence information was incomplete. Additionally, four *Charadrius* species were missing sequence data for one or two genes, namely *C. bicinctus, C. placidus, 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.

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

Gene	Length (bp)		Nucleotide frequencies (%)		Transition/ transversion bias (*R*)	Mean pairwise identity (%)	Nucleotide substitution model
	Fresh samples	Museum samples (% of full sequence)	AT	CG			
ND3[a]	401	200 (50)	51.3	48.7	2.7	88.6	HKY + I + G
COI[a]	626	429 (69)	57.0	43.0	3.9	89.3	GTR + I + G
ADH5[b]	829	150 (18)	54.5	45.5	4.6	95.5	GTR + G
FIB7[b]	840	138 (16)	63.4	36.6	1.4	95.2	GTR + G
MYO2[b]	688	209 (30)	52.3	47.7	2.5	96.4	HKY + G
RAG1[b]	911	217 (24)	53.4	46.6	4.1	98.2	HKY + I + G

[a] mitochondrial loci; [b] nuclear loci

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

	COI	*ND3*	*ADH5*	*FIB7*	*MYO2*	*RAG1*
ND3[a]	-	81.2	74.1	73.2	76.0	80.3
COI[a]	81.2	-	73.8	76.7	80.2	76.2
ADH5[b]	74.1	73.8	-	73.9	75.2	76.2
FIB7[b]	73.2	76.7	73.9	-	72.1	73.8
MYO2[b]	76.0	80.2	75.2	72.1	-	75.8
RAG1[b]	80.3	76.2	76.2	73.8	75.8	-

[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.

Gene	All			1st codon			2nd codon			3rd codon		
	I_D	χ^2	p	I_D	χ^2	p	I_D	χ^2	p	I_D	χ^2	p
COI[a]	0.012	24.87	>0.99	0.034	13.79	>0.99	0.011	21.34	>0.99	0	37.15	>0.99
ND3[a]	0.026	13.8	>0.99	0.075	31.57	>0.99	0.006	10.93	>0.99	0.005	4.08	>0.99
ADH5[b]	0.006	34.8	>0.99	0.013	23	>0.99	0.01	17.74	>0.99	0.005	45.03	>0.99
FIB7[b]	0.006	24.3	>0.99	0.001	17.59	>0.99	0.01	15.69	>0.99	0.013	77	0.95
MYO2[b]	0.00	80.16	0.88	0.00	37.11	>0.99	0.00	46.92	>0.99	0.00	53.86	>0.99
RAG1[b]	0.004	22.39	>0.99	0.00	9.41	>0.99	0.006	18.69	>0.99	0.00	18.67	>0.99

[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.

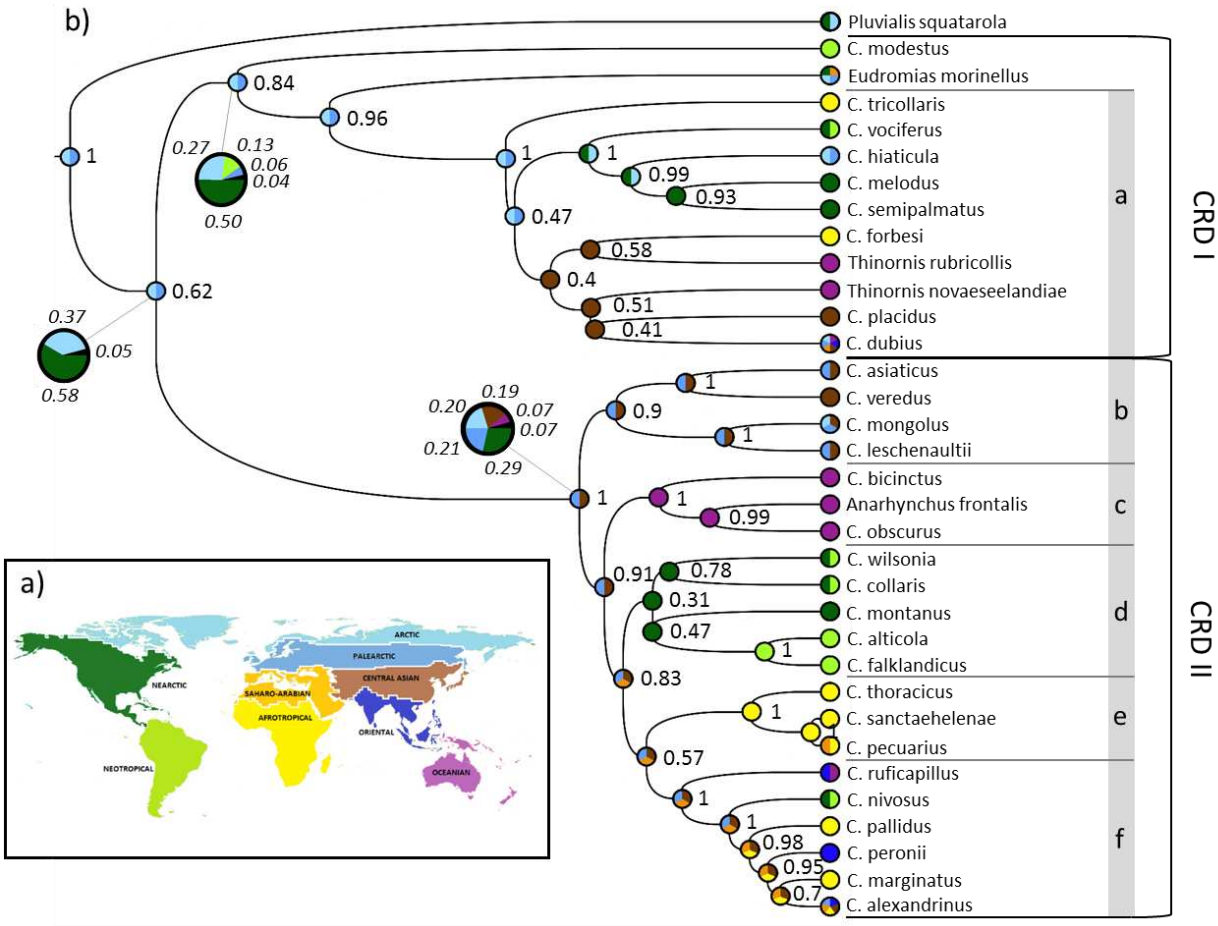

Figure 2: *BEAST maximum clade credibility trees based on sequence data from six genes: a) *COI*[*], b) *ND3*[*], c) *ADH5*, d) *FIB7*, e) *MYO2* and f) *RAG1*. [*]Mitochondrial gene. Branches are shaded according to posterior probability. Black species labels indicate matches to MCC species tree (Fig. 1); grey and red labels indicate differences to MCC species tree placement, either within or between minor clades respectively.