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Re-evaluation of the taxonomic status of the ‘diminutive’ Kentish Plover Charadrius alexandrinus seebohmi based on phenotypic and genetic analyses

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Re-evaluation of the taxonomic status of the ‘diminutive’ Kentish Plover *Charadrius alexandrinus seebohmi* based on phenotypic and genetic analyses

ABSTRACT

The Kentish Plover (*Charadrius alexandrinus* Linnaeus 1758) is a common shorebird of Eurasia and North Africa that breeds in a variety of ecological habitats, exhibits a different extent of migratory behaviour, and is an emerging model species of breeding system evolution. Here, we focus on *C. a. seebohmi* (Hartert & Jackson 1915), a resident population found in the southern tip of India and Sri Lanka, and re-evaluate its taxonomic status based on phenotypic and genetic distinctiveness, from its sympatric, migrant conspecific – *C. a. alexandrinus* and the recently elevated closely related species, *C. dealbatus* in East Asia. We show that *C. a. seebohmi* has different body size, moulting pattern and plumage colouration from both conspecifics. Furthermore, based on 2 mitochondrial, 2 sex-linked and 11 autosomal microsatellite markers from 28 *C. a. seebohmi*, 219 *C. a. alexandrinus* and 131 *C. dealbatus* individuals, we show that these three taxa have moderate genetic differentiation (F_{st} = 0.078 - 0.096), and *C. a. seebohmi* is sister to the clade of *C. a. alexandrinus* and *C. dealbatus* with an estimated 1.34-million-year divergence. In addition, we examined ornithological records of major museum collections of C. *a. seebohmi* in Asia, Europe and North America, to evaluate its range and taxonomic status. Based on the genetic, phenotypic, allochronic migratory pattern and breeding range, we propose species status for *C. a. seebohmi* as *Charadrius seebohmi* (Hartert & Jackson 1915) with the proposed English name 'Hanuman Plover'.

KEYWORDS

Hanuman Plover, isolation, South Asia, Sri Lanka, subspecies, Shorebirds

INTRODUCTION

Species with broad geographical ranges are exposed to a variety of physical barriers and environmental heterogeneities, therefore have the tendency of population sub-structuring (Peñalba *et al.* 2019, Liu *et al.*, 2020). Such taxa tend to consist of multiple geographical populations that have distinct morphological characters, and some deserve species or subspecies status (Price 2008). Subspecies could exhibit substantial genetic differentiation and eventually diverge into full species due to reproductive isolation. Barn Swallow (*Hirundo rustica*), gulls (*Larus*) and wagtails (*Motacilla*:) are several such examples (Harris *et al.* 2018, Scodato and Safron 2014, Liebers-Helbig *et al.* 2001).

The Kentish Plover (*Charadrius alexandrinus*) is a cosmopolitan species complex, previously consisting of four subspecies: *C. a. alexandrinus* Linnaeus 1758 (Linnæi & Salvii 1758), *C. a. nivosus* Cassin,1858 (Oberholser, 1922), *C. a. dealbatus* (Swinhoe 1870) and *C. a. seebohmi* (Hartert & Jackson 1915), separated geographically with some subtle morphological and plumage variations (del Hoyo *et al.* 1996, Kennerley *et al.* 2008, del Hoyo *et al.* 2021). Historically, White-fronted Plover *Charadrius marginatus* was also considered a subspecies of Kentish Plover (e.g., Roberts 1940, Mackworth-Praed and Grant. 1960). Geographical barriers, like islands, impact the level of genetic diversity and population genetic differentiation in this species complex (Küpper *et al.* 2009, 2012; Almalki *et al.* 2016; Küpper & dos Remedios 2019). Recent studies evaluated taxonomic status of some of these subspecies and proposed the elevation of the Snowy Plover *C. nivosus* (Küpper *et al.* 2009) and White-faced Plover *C. a. dealbatus* (Rheindt *et al.* 2011, Sadanandan *et al.* 2019, Wang *et al.* 2019a,b) as full species, based on their genetic and phenotypic distinctiveness, which has now been widely recognized (e.g., del Hoyo *et al*. 2021; Gill *et al.* 2021).

The wide geographic distribution of *C. alexandrinus* which includes inland lakes, grasslands, high plateaus, coastal beaches, and Salinas (del Hoyo *et al.* 2021, Meiniger & Székely 1997) and the fact that it breeds on several offshore and oceanic islands (e.g., Canary Islands, Azores, Cape Verde; AlMalki *et al*. 2016) provide excellent opportunities for testing speciation hypotheses. Furthermore, *C. alexandrinus* hybridises with *C. dealbatus*, and understanding this ongoing process provides us understanding of the role of genetic introgression in species diversification (Wang *et al*., 2019a). *C. alexandrinus* is also an emerging model species for breeding system evolution (Székely 2019), since different populations often exhibit different mating systems and parenting, and a series of experimental and observational studies have uncovered the evolutionary costs & benefits and neuroendocrine bases of these different strategies (Kosztolányi *et al*. 2012, Carmona *et al*. 2015, Eberhart-Phillips *et al*. 2017, Song *et al*. 2020).

Here we focus on a diminutive non-migratory form of *C. alexandrinus,* found in Sri Lanka and the Palk Bay region of south India (Seebohm 1887), which is considered a distinct subspecies based on the differences in plumage and behaviour (Hartert & Jackson 1915; Figure 1). *C. alexandrinus,* breeding in the northern latitudes of Asia (approximately above 40°), migrates to equatorial regions in the non-breeding season (Küpper *et al*. 2009); so both migratory (*C. a. alexandrinus*) and resident (*C. a. seebohmi*) populations are found therein, during the non-breeding season. However, in the breeding season (in May-July), only the resident *C. a. seebohmi* breeds in these equatorial latitudes (del Hoyo *et al.* 2021, Rasmussen and Anderton 2005, Henry 1998, Ali and Ripley 1983).

The taxonomic history of *C. a. seebohmi* is convoluted. In 1872, E. H. W. Holdsworth identified small plovers in his collection from Sri Lanka as ‘young’ *Ægialites cantianus* (= *C. alexandrinus*, Holdsworth 1872). This population of *C. alexandrinus* was recognized as a separate taxon, *C. cantianus minutus* by Seebohm (1887). Since *C.*

cantianus minutus (Seebohm 1887) and *C. minutus* (Pallas 1831, referring to *C. dubius* – Little Ringed Plover) were homonyms and hence invalid, Hartert and Jackson (1915) proposed a *nomen novum* (a new name) for this taxon, *C. a. seebohmi,* by assigning an adult male from the Holdsworth Collection in the Rothschild Museum, Tring, UK (now at the American Museum of Natural History; AMNH 736757), collected from "Aripo, Ceylon" (= Arippu, Sri Lanka) on 04 March 1869 as the holotype (Holdsworth 1872, Hartert & Jackson 1915). A male specimen of this same plover, from V. Legge (Natural History Museum, UK; NHMUK 1896.7.1.545), collected on 27 June 1873 from the Southern coast of Sri Lanka (Hambanthota), was described as *Leucopolius alexandrinus leggei* (Whistler & Kinnear 1936), a junior synonym of *C. a. seebohmi* Hartert and Jackson 1915 (Rasmussen & Anderton 2005, Pollock 2015).

Besides the plumage and morphological differences (Seebohm 1887, Hartert & Jackson 1915, Whistler & Kinnear 1936, Ali and Ripley 1983, Rasmussen and Anderton 2005), preliminary field studies suggest that *C. a. seebohmi* in Sri Lanka also has distinctive courtship displays and vocalizations (Seneviratne unpublished data and vocal recordings; eBird accession numbers: ML 415770261, ML 415770281, ML 415770291, ML 415770321, ML 415770331, ML 415770341). These differences suggest that *C. a. seebohmi* may be a distinctive lineage of *C. alexandrinus,* and could be considered as a full species.

To fill this gap in the taxonomy and biogeography of the *C. alexandrinus* complex in South Asia, here we critically assess the level of divergence in morphometrics, plumage and genetics, using mitochondrial, sex-linked loci and autosomal microsatellite markers, for a sample set combined of *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus*. In addition, we also examined the historical literature and the specimens in museum collections, to elucidate the conflict in nomenclature and to evaluate the taxonomic position of *C. a. seebohmi*.

METHODS

Sample and data collection

We captured and took blood samples from seven female and six male nesting *C. a. seebohmi* from Mannar Island (near the type locality, Arrippu), Sri Lanka (9°05'15.7" N, 79°41'58.6" E and 9°04'37.4" N, 79°47'56.6" E), between May and August 2019. Another six blood samples with morphometric data were collected alongside the National Bird Ringing Programme (NBRP), in June and August between 2013 and 2020, from Bundala National Park (Hambanthota), Sri Lanka (6° 10' 52.62” N, 81° 14' 5.46” E) (Figure 2). Sampling was carried out using mist nets and funnel traps (as described in Karr 1981, Székely *et al.* 2007), and the captured birds were processed at the capture location and released immediately with a coloured leg band for later behavioural observations (Hjort & Lindholm 1978).

A 50 µL sample of blood was collected from the brachial vein and samples were stored in cryogenic vials filled with 500 µL of Queen’s lysis buffer (Owen 2011). Measurements of birds (mass, head length, bill length (culmen), bill height and width, length of the tarsus, flattened wing length, tail length, and length of each primary, secondary and tertiary feather of left wing) were taken with a Vernier calliper (± 0.02 mm), a wing ruler (± 0.1 mm) and a Newton scale (±1.0 g) as in Seneviratne *et al.* (2012). Biometric and genetic data from 11 populations of *C. a. alexandrinus* and eight populations of *C. dealbatus* were obtained from the Chinese coast, Qinghai Lake and Taiwan (Wang *et al.* 2019b). In addition to the measurements from live specimens, key biometric data from 12 museum specimens of *C. a. seebohmi* including six males and six females were obtained from the National Museum of Natural History, Sri Lanka (NMSL). Subspecies level identification was carried out considering the differentiation of plumage characters (colour of loral stripe, facial plumage and crown) and the wing length (mean: 101.4, range: 96-106 mm), as described in Kennerley *et al.* (2008), Rasmussen & Anderton (2005) and Hartert and Jackson (1915).

Phenotypic data collection and analysis

Morphometric data of 31 *C. a. seebohmi,* 379 *C. a. alexandrinus* and 163 *C. dealbatus* were used in phenotypic analysis. To compare the body dimensions, analyses of variance (ANOVA) and Tukey's honest significant difference (Tukey 1949) were used. A principal component analysis (PCA) was carried out using four biometrics (wing length, bill length, tail length and tarsus length) to visualize the biometric space of the three taxa. Individuals with complete sets of measurements were used in this analysis. Plumage comparisons were conducted using the same set of individuals (above). Due to the possible differences in the patterns of moulting in *C. seebohmi*, plumage variables were not analysed quantitatively (see Results).

Biometrics were used in a species delimitation criterion (Tobias *et al.* 2010) to determine the phenotypic rigor of the taxonomic status of *C. a. seebohmi*. Cohen's *d* effect size (Cohen 1988) was calculated using mean of biometrics to determine the magnitude score of each biometric measurement (Tobias *et al.* 2010). In addition to the known plumage variations, comparisons of photographs published by the Oriental Bird Club (http://orientalbirdimages.org/birdimages.php; n = 20) and eBird (https://ebird.org/; n = 20) were used to differentiate plumage characters.

Data from 12 *C. a. seebohmi* nests were used to assess the breeding behaviour in compared to *C. a. alexandrinus*. In addition, calls of *C. a. seebohmi* (n = 10) and *C. a. alexandrinus* (n = 12) were retrieved from Xeno-Canto (https://www.xeno-canto.org) and Macauley Library of Natural Sounds (https://www.macaulaylibrary.org/) to study the vocal differentiations between two taxa.

All statistical analyses were conducted in R version 4.0.5 (R Core Team 2021) using RStudio, version 2021.09.1+372 (RStudio Team 2020).

DNA Sequencing and genotyping

Genomic DNA was extracted using the phenol-chloroform DNA extraction method (Burke & Bruford 1987). Extracted DNA was visualized in 0.5% agarose gel in TAE buffer with ethidium bromide (EtBr, 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide) using an ENDURO™ GDS Gel Documentation System.

Blood samples from breeding males from Sri Lanka were used for the genetic analysis to ensure that the *C. a. seebohmi* genotype is considered for the analysis. Extracted DNA of 10 male *C. a. seebohmi* from Mannar (n = 5) and Bundala (n = 5) were used to amplify a total of 19 genetic markers including two mitochondrial loci – Partial D-loop of the mitochondrial control region (CR), NADH dehydrogenase subunit 3 (ND3) and two z chromosome-linked regions; Z4 and Z45, and 11 autosomal microsatellite loci including Calex 01, 04, 05, 08, 11,18, 19, 22, 23, 32, 37, 39 and 45 (Küpper *et al.* 2007), C204 (Funk *et al.* 2007) and Hru2 (Primmer *et al.* 1995) following lab protocol described in Wang *et al.* (2019b) with reagents and optimizations from Wenink *et al.* (1994), Chesser (1999), Funk *et al.* (2007), Küpper *et al.* (2007) and Ji *et al.* (2014). PCR reactions were carried out using a MultiGene™ OptiMax Thermal Cycler (Labnet International, Inc., New Jersey, USA). The 10 successfully amplified individuals were sequenced from Macrogen, Inc. (Seoul, South Korea). Raw chromatograms were manually checked and edited using Chromas (version 2.6.6) (McCarthy 1997). Microsatellite genotyping was carried out using capillary gel electrophoresis in an ABI

3500 dx (Applied Biosystems, Massachusetts, USA). Individual microsatellite traces and peak calls in electropherogram files were analysed using Geneious; version 1.4.6 (Kearse *et al.* 2012) along with a Microsatellite Analysis External Plug-in.

We compared our dataset with Wang *et al.* (2019a), which included 219 *C. a. alexandrinus* from 11 populations and 131 *C. dealbatus* from eight populations along the Chinese coast and Taiwan. All the laboratory work for this study was carried out at the Laboratory for Molecular Ecology and Evolution at the Department of Zoology and Environment Sciences, University of Colombo.

Phylogenetic analysis

To determine the phylogenetic position of *C. a. seebohmi*, we used amplified samples of CR, ND3, Z4 and Z45 (see above) and additional sequences from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/; numbers MK830765-MK830815, MK830754-MK830764, AM941556-AM941555, and KM001420-KM001422) representing members of the *C. alexandrinus* complex (Küpper *et al.* 2009, Rheindt *et al.* 2011, Wang *et al.* 2019b)□. *C. pecuarius* was used as the outgroup. Final concatenated phylogenetic trees and haplotype networks were built using selected sequences from pre-built consensus trees for each region. Sequences were loaded into MEGA (version 7.0.26: Kumar *et al.* 2016) and nucleotides were aligned using the MUSCLE algorithm (Edgar 2004). Aligned nucleotide sequences were revised into protein coding codon sequence to ensure maximum reliability for the sequence (Wickremasinghe *et al*. 2017). Concatenated phylogenetic trees were built in both Maximum Likelihood (ML) and Bayesian approaches. ML tree was built using IQ-TREE (version 1.6.12) with 10,000 standard bootstrap replicates (Nguyen *et al.* 2014). A Bayesian tree was built using MrBayes (version 3.2) with two simultaneous 1,000,000 generations (Ronquist *et al.* 2012), with

25% of first trees burnt-out to obtain more accurate posterior probabilities. This Bayesian inferred execution was based on Markov Chain Monte Carlo (MCMC) analysis (Sorensen & Gianola 2007). Best-fit models for gene partitions were investigated using PartitionFinder (Version 1.1.0: Lanfear *et al.* 2012) according to Bayesian Information Criterion (BIC) instead of Akaike Information Criterion (AIC) due to lower complexity in the dataset (Chen & Chen 2008). FigTree (version 1.4.4) was used to visualize the phylogenetic trees (Andrew 2006). Further, we built haplotype networks for all four loci (CR, ND3, Z4 and Z45) separately according to the median joining network algorithm (Bandelt *et al.* 1999) in PopArt (version 1.7) with the epsilon (ε) value of 0 (Leigh & Bryant 2015). Sequences from CR, ND3 and Z-chromosome linked Z4 and Z45 regions of this study, Rheindt *et al.* (2011) and Wang *et al.* (2019b) were used to build haplotype maps to represent genetic variation among closely related *Charadrius* plovers.

Divergence time estimation

A time calibrated phylogeny was reconstructed using the CR region in BEAST 2.5.1 (Drummond and Bouckaert 2015). We tested the substitution model of the CR gene with jModelTest 2.1.4 based on Bayesian Information Criterion (Darriba *et al.* 2012). The best model HKY+I was implemented in the BEAST analyses. A Yule model was selected as the tree prior, and relaxed uncorrelated lognormal distribution was used as clock model. The clock rates were set at 0.013 substitution/site/million year based on the rate estimated for the mitochondrial Control Region (Weir & Schluter 2008). The chain length of MCMC runs was set at 10 million generations, with sampling frequency of every 1000 generations. Two independent runs were performed. Tracer v1.7 (Rambaut *et al.* 2018) was used to assess convergence among the two independent runs and to confirm adequate effective sample sizes (ESS) of the parameters (ESS ≥ 200). The two runs were then combined using LogCombiner v2.4.7 (Drummond and Bouckaert 2015) with 20% burn-in. Finally, the trees were summarized to produce a maximum clade credibility tree with TreeAnnotator v1.8.2 (Rambaut and Drummond 2007).

Population genetic analysis

Using the 11 autosomal microsatellite loci, Bayesian clustering was performed in STRUCTURE (version 2.3.4; Jonathan *et al.* 2000) to identify genetic clustering among the three taxa. STRUCTURE runs were performed as individual simulations with a manual input of population number (K) from 1 to 10 in 100,000 MCMC runs with burn-in of 10,000. Each K was iterated 10 times to increase accuracy and to obtain ΔK to determine the best K value (Evanno *et al.* 2005, Falush *et al*. 2003). Structure Harvester (version 0.6.94; Earl & VonHoldt 2012) was used to get the best population number. A final run under the best K value was performed again in STRUCTURE software with 1,000,000 MCMC runs with burn-in of 100,000 (Rheindt *et al.* 2011). A second run with the known number of populations (K = 3) was also performed (Figure 2) with results illustrated as bar plots using Distruct: version 1.1 (Rosenberg 2004)□.

GenAlex; version 6.5 (Peakall & Smouse 2006) was used to calculate pairwise Fst values using Weir and Cockerham (1984) genetic distance. Significance was assessed based on 1000 permutations with significance levels adjusted for multiple testing using sequential Bonferroni procedure. All the figures were retraced and illustrated using Inkscape; version 1.1 (Bah 2007).

RESULTS

Morphometric analysis

Biometrics clustered into three distinct groups in the PCA (Figure 3e), and the first three principal components explained 98% of the variance (Table S1).

Breeding *C. a. seebohmi* adults of both sexes (Figure 1) were smaller than *C. a. alexandrinus* and *C. dealbatus* in bill length, tarsus length, wing length and tail length (ANOVA: $F_{2,\,138} = 317.05$, $P < 0.001$; Table 1). *C. a. seebohmi* had shorter wings than that of *C. a. alexandrinus* (Tukey-Kramer Test $P < 0.001$, 95% C.I. = -15.80, -12.79), and *C. dealbatus* ($P = 0.000$, 95% C.I. = -18.12, -14.95; Table 1). Since the assumption of homogeneity of variance was not met for the tarsus, bill, and tail lengths, we used Welch's adjusted F ratios for those comparisons of phenotypic traits. The tarsus length of *C. dealbatus* was longer than that of the other two studied species ($F_{2,\,46.56} = 1845.05$, $P < 0.001$; Table 1). *C. a. seebohmi* had the shortest tarsus (Games-Howell test; $P < 0.001$, 95% C.I. = -3.38, -1.56; Table 1).

Bill length was longer in *C. dealbatus* ($F_{(2,\,75.64)} = 88.95$, $P < 0.001$), but not different between *C. a. seebohmi* and *C. a. alexandrinus* (Games-Howell Test; $P = 0.939$, 95% C.I. = -0.65, 0.85). Similarly, the tail length differs only in *C. dealbatus* (Welch's $F_{2,\,56.59} = 1942.79$, $P < 0.001$), while it did not differ between *C. a. seebohmi* and *C. a. alexandrinus* (Games-Howell Test; $P = 0.165$, 95% C.I. = -7.85, 1.08; Table 1, Figure 3).Three separate clusters, one for each taxon, were visualized in PCA (Figure 3 e) where the first three principal components explained 98% of the variance (Supplementary Table 1). The species delimitation based on phenotype showed a magnitude value of 16 (Table 2).

Plumage and behaviour

It appears that, unlike *C. a. alexandrinus* and *C. dealbatus*, *C. a. seebohmi* does not have a rufous cap during the breeding season. The adult breeding male *C. a. seebohmi* has rufous buff mixed in with its paler cap, dark incomplete breast patches (complete in *C. a. alexandrinus*) and a black forehead stripe. These breast patches and forehead stripe become paler in the non-breeding plumage. *C. a. seebohmi* also has an incomplete front eye-lore which becomes darker in the breeding plumage. It has dark grey legs in both sexes (*C. a. alexandrinus* has black legs). Female *C. a. seebohmi* resembles female *C. a. alexandrinus*, but lacks the warm tone in the forehead stripe, ear coverts, front eye-lore and breast patches in breeding plumage.

Moult patterns

Our observations based on photos of banded birds suggest that the moulting pattern of *C. a. seebohmi* is more close to that of *C. dealbatus*, likely driven by their similar non migratory behaviour (Jonathan Martinez, pers. Obs.). They apparently have a very early moult into breeding plumage type, very likely during the autumn season and thus much earlier than any *C. a. alexandrinus*. Birds are starting moulting into nonbreeding plumage just before breeding in April and seems to suspend this moult as they start breeding. This suspended moult seems to be variable, and some flight feathers can be already moulted before they starts breeding in some individuals. This suspended moult allows them to acquire partial non-breeding plumage before the breeding season (Figure 1), in the same manner of *C. dealbatus*, but in the latter only contour feathers are concerned (Jonathan Martinez, in prep.) .

Vocalization

On average, *C. a. Seebohmi* has higher number of notes (4) and higher frequency (2081Hz) in vocalization than that of *C. a. alexandrinus* (2.9 and 1107Hz respectively: Table 2). Therefore buzzy '*tszwEET*' and '*TreiuT*' calls of *C. a. seebohmi* is slightly pitchy and of higher frequency (Macaulay Library Accession Numbers; ML 415770261, ML 415770281, ML 415770291, ML 415770321, ML 415770331, ML 415770341). Aerial display and flight call at breeding grounds are uncommon in *C. a. seebohmi* (see ML 415770281 and ML 415770331 for examples of aerial display).

Taking all phenotypic data together, we obtained a magnitude value of 16 (Table 2), which qualifies *C. a. seebohmi* for species status (Tobias *et al.* 2010).

Genetic analysis

A concatenated dataset of 927 bases from CR and ND3 regions fully supported a separation of *C. a. seebohmi* from *C. a. alexandrinus* with a bootstrap value of 100 in the ML tree and a posterior probability value of 0.9976 in the Bayesian tree (Figure 4). We used sequences from 345 individuals of *C. alexandrinus* and *C. dealbatus* across their range, and gradually reduced the number of sequences to avoid polytomy formation between *C. a. alexandrinus* and *C. dealbatus*. Within the *C. alexandrinus* species complex, *C. a. seebohmi* is sister to the *C. a. alexandrinus* and *C. dealbatus* sister pair (Figure 4). Divergence time estimation for *C. a. seebohmi* from *C. a. alexandrinus* using sequences of CR was estimated as 1.2 (95% Highest Posterior Density: 0.46 – 2.10) million years ago.

Haplotype networks for *C. a. seebohmi* and four other closely related taxa in the *C. alexandrinus* complex showed a clear and distinct divergence of *C. a. seebohmi* in the CR region (Figure S1) with a nucleotide diversity (π) of 0.02907. The less divergent ND3 region showed the least separation (π = 0.01645). A concatenated sequence of CR, ND3, Z4 and Z45 of 2327 bp had a nucleotide diversity (π) of 0.00184. The level of genetic polymorphism of *C. a. seebohmi* against its closely related taxa is summarized in Table S2.

About 16 variations in allele number repeats (repeat motifs) were seen among the three taxa where *C. a. seebohmi* was separated from *C. a. alexandrinus* and *C. dealbatus* (Table S3). Genetic cluster analysis in STRUCTURE segregated *C. a. seebohmi* into a distinct cluster from *C. a. alexandrinus* and *C. dealbatus* (for both $K = 3$ and $K = 4$) (Figure 2). Results of pairwise F_{ST} between groups (0.078-0.095: adjusted $P < 0.01$) showed a moderate genetic differentiation of *C. a. seebohmi* compared to *C. a. alexandrinus* and *C. dealbatus* which isolates *C. a. seebohmi* from other members of the *C. alexandrinus* cluster (Table 3).

DISCUSSION

Using morphometric measurements and plumage characteristics, breeding distribution, molecular phylogeny based on mitochondrial and autosomal markers and Bayesian population clustering based on 11 microsatellite markers, we demonstrated the phenotypic and genetic distinctiveness of the resident population of Kentish Plover, *C. a. seebohmi*, of Sri Lanka and the Palk Bay region of southern India. Its sister taxa, the widespread Kentish Plover, *C. a. alexandrinus,* and the White-faced Plover, *C. dealbatus*, are larger in size, different in plumage and genetic signature, and do not share the breeding ranges with *C. a. seebohmi*. The small size, shorter wing length, the different moult strategy and timing, and lack of rufus crown and broken loral stripe in breeding season, separate *C. a. seebohmi* from its congeners in the field (Figure 1).

These results are important for three reasons. First, it is showing another evidence of cryptic speciation in the genus *Charadrius* (Küpper & dos Remedios 2019, Wang *et al.* 2019a, Wei *et al*. 2022), presenting a likely case of incipient divergence probably due to allochronic segregation in breeding time (Taylor & Friesen *et al*. 2017, Tang *et al*. 2022). Second, based on the substantial genetic and phenotypic differences between *C. a. seebohmi* and other *Charadrius* populations, we suspect that the breeding ecology of *C. a. seebohmi* to be different from the well-studied European and Chinese plover populations (Wang *et al*. 2019a, Székely 2019, Sadanandan *et al.* 2019, AlMalki *et al*. 2016). Thus, there is a need to carry out detailed behavioural, ecological and morphological studies of this population. Third, from a broader perspective, endemic and range restricted taxa in the Global South are a major asset in defining and managing natural resources that are unequally distributed (IRP 2019)□. The recognition of such unique biota, therefore, is a vital first step in biodiversity conservation (Avise 2000), because such taxa can act as flagship species to draw much needed conservation attention for critical ecosystems□ (BirdLife International 2004).

Phenotypic patterns

The body weight, bill length and tarsus length are often indicators of dominance or reproductive advantages in smaller plovers (Sadanandan *et al.* 2019). For example, *C. dealbatus* is bigger and might displace smaller *C. alexandrinus* in the breeding season, which could provide partial reproductive isolation (Rheindt & Edwards 2011, Sadanandan *et al.* 2019)□. The smaller body size of *C. a. seebohmi* (Rheindt *et al.* 2011) could be attributed to less intense sexual selection as suggested for the latitudinal variation in size and selection pressures in other plovers (Ji *et al.* 2014, Wang *et al.* 2019a). Allen's (Allen 1877) and Bergmann's (Meiri and Dayan 2003) rules suggest that animals in higher latitudes tend to be larger with relatively shorter protruding appendages to counter higher physiological demands of high latitudes. The smaller size of tropical breeding Kentish Plovers in south Asia and tropical Africa could be simply due to the relaxation of such physiological demands. The shorter legs and bill of *C. a. seebohmi* is indicative of allometry of smaller overall size. Its shorter wings, however, could be attributable to the non-migratory behaviour of the tropical population as long-distance migrants have longer primary flight feathers to increase forward propulsion (Lovette & Fitzpatrick 2016, Hera *et al.* 2020)□.

C. a. alexandrinus starts breeding in mid-April to May in higher latitudes (Northwest Europe), but in tropical regions in Africa it breeds in as early as mid-November (e.g., on Socotra Island; Porter & Suleiman 2014), January (Cape Verde; Snow & Perrins 1998), February (Northeast Africa) and in March (Northwest Africa and Iraq; Hanane 2011, del Hoyo *et al.* 2021); therefore the earlier transition to breeding plumage might be an adaptation of C. *alexandrinus* in tropical latitudes. Tropical breeding *C. dealbatus* moults into breeding plumage as early as October (Kennerley *et al.* 2008). The moulting strategy of *C. a. seebohmi* differs from that of *C. a. alexandrinus* (JM unpublished data), therefore comparisons of breeding and non-breeding plumages are not feasible with the available anecdotal information on their moulting. Our observations suggest that the moulting pattern in *C. a. seebohmi* is like that of *C. dealbatus* (Kennerley *et al.* 2008). It starts moulting into nonbreeding plumage just before breeding in April. However, unlike the latter (del Hoyo *et al.* 2021), it appears to have a suspended primary moult in June from which they acquire partial non-breeding-like plumage before the breeding season. Therefore, *C. a. seebohmi* moulting into non-breeding plumage led to the fading of 'ornamentation' or 'contrast' observed, especially in males.

The disappearance of colourful feather tracts, especially in males of island populations, had been observed in several species such as White-winged Fairywrens (*Malurus leucopterus*; Doucet *et al.* 2004) and Common Redshanks *(Tringa totanus)* (Fitzpatrick 1998)□. The forces of sexual selection that are mostly responsible for ornamental plumage patterns (Andersson 1994) could be reduced in island and tropical birds partly due to the longer and less defined breeding seasons (Sinclair 1978, Diamond 1977). However, longer breeding seasons in tropics could intensify selection pressure in some groups through multiple breeding cycles or through polygamy (Lande 1976, Ausband 2018). Fading of colours in longer wavelengths due to the intense solar radiation in tropics could also be a factor in reduction of colour in tropical breeders (Tickell 2003). The different predator assemblages and vegetation profiles could also contribute to this fading of coloration (Schluter & Conte 2009, Hubbell 1997).

Potential drivers of divergence in *C. a. seebohmi*

The closest relatives of *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus* diverged at about 0.6 Mya (Wang *et al.* 2019b, Sadanandan *et al.* 2019). Despite their genetic and phenotypic distinctiveness (Wang *et al.* 2019a), they have a considerable amount of gene flow in sympatry (Wang *et al*. 2019b), hence, a narrow hybrid zone emerged on the south-eastern Chinese coast (Sadanandan *et al.* 2019, Wang *et al.* 2019b). The separation of *C. a. seebohmi* from its sister clade had taken place much earlier, at about 1.34 Mya (Figure 4). The climatic conditions of the mid-Pleistocene (Berger & Jansen 1994) might have triggered this divergence in the ancestral continental populations in South Asia as shown in other avian groups in the region (Wickramasinghe *et al.* 2017, Jha *et al*. 2021). Like its East Asian sister pair, the disjunct breeding distribution of South Asian pair (*C. a. alexandrinus* and *C. a. seebohmi*) suggests spatial segregation in breeding range. The sympatry in non-breeding (winter) months and the occurrence of non-breeding *C. a. alexandrinus* as ‘summer loiterers’ in the tropical breeding grounds of *C. a. seebohmi*, however, suggest a possibility for introgression between these phylogenetically distinct taxa. A detailed future study aiming at much broader sampling of these seemingly allopatric plovers in Sri Lanka and south-central India would shed more light into the population genetic structure of *C. a. seebohmi*.

Allopatric speciation, in which physical barriers restrict gene flow between two populations, facilitating the initiation of divergence through selection (Mayr 1963)□ or genetic drift (Coyne & Price 2000)□, is the prevalent mode of speciation in birds (Price 2008). If populations remain isolated for a long enough period after divergence has been established, genome-wide differentiation can be accumulated (Nosil & Schluter 2011). The accumulation of genomic changes can be drastic or subtle, yet such changes, even in narrow genomic tracts, can contribute to strong reproductive isolation (Grossen *et al.* 2016). Allopatry due to migratory divides or allochronic difference in migratory behaviour, therefore, would contribute prezygotic isolation and divergence (Delmore *et al.* 2016, Taylor & Friesen *et al*. 2017, van Bemmelen *et al.* 2019).

Wang *et al.* (2019b) found genetic and phenotypic distinctiveness along the Chinese coast in the closest relatives of *C. a. seebohmi*. The divergence of *C. dealbatus* from *C. alexandrinus* had been facilitated by sea level fluctuation in the Pleistocene, character displacement and associated ecological niche differentiation (Wang *et al.* 2019a)□. Sexually mediated traits such as plumage colouration aimed at mate attraction (Andersson 1994) can act as key divergent forces in reinforcing such speciation events (Price 2008). Hindrance of gene flow through allopatry can also play a role. As suggested by previous morphometric (Kennerley *et al.* 2008) and genetic studies (Sadanandan *et al.* 2019), these plovers maintain allopatry in breeding ranges. The breeding population of *C.*

a. seebohmi, for example, is separated from the closest breeding populations of *C. a. alexandrinus* by ~1200 km and *C. dealbatus* by about ~1000 km (del Hoyo *et al.* 1992).

C. alexandrinus exhibits high gene flow due to dispersal between breeding sites (Küpper *et al.* 2012). Lack of dispersal is key to hindrance of gene flow (Funk *et al.* 2007) where *C. a. seebohmi* is severely limited, first by the latitudinal segregation and then by the limited linear coastal habitat in Sri Lanka and southern tip of peninsula India. Since the two subspecies do not overlap in the breeding season, *C. a. seebohmi* does not show a strong parapatric affinity to *C. a. alexandrinus*. Local adaptations might have further limited dispersal and facilitated divergence (Rheindt & Edwards 2011).

Taxonomic redescription

Charadrius seebohmi (Hartert and Jackson 1915)

Charadrius cantianus Latham 1801

Charadrius cantianus minutus Seebohm 1887 (not *Charadrius minutus* Pallas 1831)

Charadrius alexandrinus seebohmi Hartert and Jackson 1915

Leucopolius alexandrinus leggei Whistler and Kinnear 1936

Holotype

AMNH 736757, male, collected from Aripo, northern Ceylon [=Arripu, Sri Lanka: 8°47'48.8"N, 79°55'28.1"E], March 1869, by E.W.H. Holdsworth, from the Rothschild collection. Since Holdsworth misidentified the species as *Ægialites cantianus,* he described the specimens as young without full plumage, but with a black bill, dark brown irides, dark grey feet and paler legs. The specimen described by Hartert & Jackson was in the "Tring Museum", which at the time (1915) was Walter Rothschild's private collection. Rothschild sold his collection to the American Museum of Natural History

(New York, USA) in the 1930s, where the specimen is now located (Greenway 1978; Figure 5 a-c). The holotype has a flattened wing length of 101.3 mm.

Other type specimens

Paratypes: AMNH 736758 (wing length = 99.2 mm) – 15 January 1870, Aripo [=Arripu, Sri Lanka], female, collected by E.W.H. Holdsworth; AMNH 736759 (wing length = 94.6 mm) – 30 March 1869, "NW Ceylon" [= north-western Sri Lanka], male, collected by E.W.H. Holdsworth. A male specimen from W.W.V. Legge's collection (NHMUK 1896.7.1.545) which was collected on 27 June 1873, from Hambanthota, Sri Lanka, was designated the holotype of *Leucopolius alexandrinus leggei* (Whistler & Kinnear 1936) which is now a junior synonym of *Charadrius seebohmi.*

Other specimens of *C. seebohmi* in major collections

There are 10 specimens from Sri Lanka collected during 1873-1949, plus one of questionable origin collected by R. Meinertzhagen (Dalton 2005) at the Natural History Museum, Tring, UK (NHMUK). The National Museum of Natural History, Sri Lanka (NMSL) houses 13 skin specimens from Sri Lanka, collected during 1881 – 1979. The American Museum of Natural History, New York, USA (AMNH) carries the holotype and two paratypes from Sri Lanka. The National Museum of Natural History-Smithsonian Institution (USNM) has three skins collected by S.D. Ripley in March-June 1944 from Sri Lanka. Yale Peabody Museum (YPM) has a single specimen collected by S.D. Ripley from Sri Lanka in September 1950 (YPM ORN 20158). Royal Ontario Museum (ROM) too has a single specimen collected by Legge in November 1894 from Sri Lanka. The Bombay Natural History Society Collection (BNHS) at Mumbai, India houses a single specimen from Kerala, India, collected by Salim Ali (see Supplementary Material for details).

Etymology

The genus *Charadrius* refers to a nocturnal waterbird where *'the sight of it would cure the jaundice*' (= an open country bird exposed to sun) (Pande *et al.* 2009)□. The species name *seebohmi* represents 'of *Seebohm*', after Henry Seebohm (1832-1895), an ornithologist who first identified the breeding Sri Lankan population as a possible subspecies in his landmark publication (Seebohm 1887). The English name 'Hanuman Plover' and the vernacular Singhalese and Tamil names, හනුමන් ඔලෙවියා *(Hanuman Olaviya)* and ஹனுமன் உப்புக்கோத்தி *(Hanuman Uppukkothi)* respectively, refer to Hanuman, the ape-god of the Hindu mythology of India and northern Sri Lanka. The Rama's Bridge (the present-day sand islands that stretch from Mannar Island to Rameswaram, India), a stronghold for *C. seebohmi* near the type locality, was constructed, according to the ancient epic *Ramayan*, by Hanuman's army. To celebrate the mythology as 'stories of the people of the native land of this plover', we suggest the name 'Hanuman Plover' as the English common name for this species.

Diagnosis

Hanuman Plover (*C. seebohmi*) is a small plover compared to its congeners. It has white underparts and grey upperparts, dark grey legs and a white hind neck collar. It has an incomplete front eye-lore which becomes darker in the breeding plumage (*C. a. alexandrinus* has a complete black line). It does not have a rufous cap in the breeding season. The adult breeding male has rufous buff mixed in with its paler cap, dark incomplete breast patches and a black forehead stripe. Female *C. seebohmi* resembles female *C. a. alexandrinus*. It lacks black in the forehead stripe, ear coverts, front eye-lore and breast patches in both breeding and winter plumages. The buzzy '*tszwEET*' and '*TreiuT*' calls of *C. seebohmi* are slightly pitchy and of higher frequency than that of *C. a. alexandrinus*.

Distribution

The *C. seebohmi* is found in coastal mudflats and adjacent grasslands in arid and dry zones of Sri Lanka and the southern tip of India. In Sri Lanka it is common in Rama's Bridge and Mannar, along the coast of North-western, Northern, Eastern and Southern provinces that include areas of Chilaw, Kalpitiya, Veddithalathive, Irranathivu, Jaffna Peninsula, islands off Jaffna, Chundikulum National Park, Mullativu, Trincomalee, Batticaloa, Kumana and Yala National Parks, and Hambanthota wetlands including Bundala National Park (Wijesundara *et al*. 2017). It can also be seen near large inland freshwater tanks in the dry zone (Henry 1998). In India, it is found in the coastal wetlands and grasslands of Kochi, Kerala to Chennai, Tamil Nadu including the coastal wetlands of Kollum, Rameswaram, Point Calemere, Cuddalore and Chengalpattu. Breeding has been reported in inland wetlands such as Vidarbha Maharashtra (Kasambe 2007) and Vani Vilasapuram, Karnataka (Rao *et al.* 2018)□.

The historical specimens that we studied in collections from 1869 to 1980 (Supplementary Table 3) yielded *C. a. seebohmi* skins in Sri Lanka and South India, and *C. a. alexandrinus* skins from elsewhere. All the putative *C. a. seebohmi* specimens collected outside their current geographic range (e.g., YPM 42366 & YPM 42367 from Bihar (North India) and UMMZ 76855 from Sind (Pakistan)) ended up being *C. a. alexandrinus* based on the plumage characteristics and wing measurements. Skins of *C. a.*

seebohmi had shorter wing lengths (mean ± SD: 100.88 ± 3.45 mm) and the identifiable plumage patterns in the face and crown (Table 1, Figure 1).

Initial reports of the ‘tropical’ ‘diminutive’ form (Seebohm 1887) of the Kentish plover referred to ‘Ceylon’ [=Sri Lanka] and south India, as well as ‘Somaliland’ [=Somalia], ‘Red Sea area’ and Djibouti (summarized in Hartert & Jackson 1915). In the subspecies description, Hartert and Jackson (1915) stated “Besides Ceylonese birds, some from Massaua [=Eritrea] and Somaliland in the British Museum undoubtedly belong to the ‘small race’”. A female from Socotra (Gulf of Yemen), considered by Sharpe (1896) as *C. a. seebohmi*, appears to be doubtful due to longer wing length (108 mm) and more contrasting facial plumage; therefore, we considered this west African specimen to be of *C. a. alexandrinus*. Based on three specimens, Friedmann (1930) stated that, “It (*C. a. seebohmi*) is confined to the coast of the Indian Ocean from Ceylon to the Mouth of the Red Sea (Somali coast). In Africa its range is restricted, as far as known, to the coast from Massawa [=Eritrea], to French Somaliland [=Djibouti]. The birds of southern Somaliland [=Somalia] are probably *C. alexandrinus*, as well as birds of Egypt, Syria, and Palestine. It is not known if *C. a. seebohmi* is resident in the Somali coast or not, as all the specimens taken are winter birds” (Friedmann 1930). According to Whistler & Kinnear (1936), the most important difference of the Sri Lankan race is that it does not acquire the chestnut cap of the breeding plumage of the nominate race. “This absence of a distinctive breeding plumage is a characteristic of other Cinghalease [=of Sri Lanka] race and is of great interest” (Whistler & Kinnear 1936). Based on that difference, Whistler and Kinnear (1936) proposed *Leucopolius alexandrines leggei* as the name for the taxon with the type being a specimen collected from Hambanthota (in Southeast coast of Sri Lanka) in 1873 (NHMUK 1896.7.1.545). However, later authors considered this nomenclature (Whistler and Kinnear 1936) as a junior synonym for *C. a. seebohmi* (Hartert and Jackson, 1915).

Since the skins from the Red Sea region came from winter months and that the morphometrics and plumage characters were aligned to *C. alexandrinus*, later authors considered the ‘Kentish Plover of tropical North Africa’ as *C. a. alexandrinus* (Mackworth-Praed & Grant 1960, Del Hoyo *et al.* 1992, Peters 1931).

Conservation implications

Sri Lanka and southern India is a global biodiversity hotspot (Myers *et al*., 2000), an area of high endemicity threatened with risk of extinction (Mittermeier *et al*., 2004). The range of *C. seebohmi* has one of the highest human population densities in the planet (MoME 2021, Luck 2007). Therefore, both breeding habitats and nonbreeding sites of the species are severely threatened with encroachment, pollution, dogs and linear infrastructure such as highways, ports and wind farms (MoME 2021). The description of *C. seebohmi* as a regional endemic could make this species a flagship species in conservation prioritization of some of the most important wetlands of the Central Asian Flyway in Sri Lanka (eg. Mannar Island, Veddithalativu Nature Reserve and Kalametiya Sanctuary). Since conservation attention is mainly driven by few charismatic megafauna and endemics in the tropics (Abeyrama and Seneviratne 2017), endemic-taxa-poor coastal wetlands such as Palk Bay region and Mannar (critical overwintering sites for migratory waterbirds; Wetlands International 2020) would greatly benefit from such regional endemics in capturing much needed conservation attention for the benefit of millions of migratory and resident shorebirds.

Acknowledgments

Author Contributions

Data Availability Statement

Upon the completion of review process: DNA sequences will be deposited at NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/), phenotypic measurement data will be deposited at Dryad database (https://datadryad.org/stash), and the vocal data will be deposited at (https://www.macaulaylibrary.org/).

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TABLES AND FIGURES

TABLE 1

Morphometric comparison of *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus* from Sri Lanka and eastern China

TABLE 2

Phenotypic character segregation of *C. a. seebohmi* and *C. a. alexandrinus*, as indicated in Tobias *et al*. (2010) character delimitation

TABLE 3

Genetic divergence among *C. a. seebohmi, C. a. alexandrinus* and *C. dealbatus*. Above diagonal: pairwise F_{ST} between groups. Below diagonal: estimates of evolutionary divergence over sequence pairs between groups. *C. nivosus* is also considered for the divergence estimates ($P < 0.01$ in all the analysis).

FIGURE 1

Adult male (A) and female (B) *C. a. seebohmi* from Mannar Island, Sri Lanka and adult male (C) and female (D) *C. a. alexandrinus* from Bohai Bay, China. Open wings of (E) *C. seebohmi* and (F) C. *alexandrinus*. Photo credits: (A-B, E: JJN, C-D: PQ, F: Chenjing Huang)

FIGURE 2

Map of Asia showing breeding range of *C. a. seebohmi*, *C. a. alexandrinus and C. dealbatus.* Inset (box) showing field sites in Sri Lanka (A) and China (B). Structure plots of Bayesian genetic clustering for three subspecies; K=4 (above) and K=3 (below) with respect to their sampling locations. Numbers represent: 1 Mannar and 2 Hambantota (Sri Lanka), 3-13 *C. a. alexandrinus* populations and 14-21 *C. dealbatus* populations (China), a-c locations of museum specimens at NMSL.

FIGURE 3

Morphometric analysis of *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus* indicated in: (A) wing length, (B) bill length, (C) tail length and (D) tarsus length of both male and female *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus*; horizontal lines within boxes represent the median values. The Principal Component Analysis (E) of *C.a. seebohmi, C.a. alexandrinus and C. daelbatus.*

FIGURE 4

Phylogenetic affinities in the *C. alexandrinus* complex generated using mitochondrial CR and ND3 regions. Values above the branches indicate bootstrap values in Maximum likelihood framework, values below indicate posterior probability in Bayesian framework.

FIGURE 5

Holotype: AMNH 736757: a male *C. a. seebohmi*, collected in 1872 from Arripu (near Mannar), Sri Lanka by E. W. H. Holdsworth. Dorsal (A), ventral (B) & side aspects (C). (Photo credit Paul R Sweet: American Museum of Natural History).

SUPPLEMENTARY MATERIAL

Museum specimen details from NMSL, ROM, NHML, Smithsonian, UMMZ, AMNH & BNHS

SUPPLEMENTARY TABLE 1

Eigenvalues and percentage of variance of the Principal Component Analysis of *C. a. seebohmi*, *C. alexandrinus* and *C. dealbatus*

SUPPLEMENTARY TABLE 2

Genetic polymorphism of *C. a. seebohmi* against its closely related taxa

SUPPLEMENTARY TABLE 3

Number of repeat motifs of different studies for 13 microsatellite loci

SUPPLEMENTARY FIGURE 1

Haplotype analysis of ND3, CR, Z4 and Z45

Tables and Figures

TABLE 1. Morphometric comparison of *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus* from Sri Lanka and eastern China. (mean ± SD)

	C. a. seebohmi (n = 23)	*C. a. alexandrinus* (n = 291)	*C. dealbatus* (n = 127)
Bill length (mm)	16.84 ± 1.67	16.74 ± 1.02	18.00 ± 1.00
Tarsus (mm)	26.10 ± 1.89	28.57 ± 1.09	50.03 ± 1.85
Wing length (mm)	100.88 ± 3.45	115.18 ± 3.18	117.41 ± 3.97
Tail length (mm)	45.96 ± 4.87	48.66 ± 2.07	28.11 ± 1.18

TABLE 2

Phenotypic character segregation of *C. a. seebohmi* and *C. a. alexandrinus*, as indicated in Tobias *et al*. (2010) character delimitation

	seebohmi	*alexandrinus*	Magnitude score	Score
Morphological (Biometric characters)	shorter wing	longer wing	effect size d = 4.31 moderate - magnitude score of 2	2
	slightly longer bill	slightly shorter bill	effect size d = 0.07 magnitude score of 0	0
	slightly shorter tail	slightly longer tail	effect size d = 0.72 minor - magnitude score of1 (co-varying with wing length)	0
	slightly shorter tarsus	slightly longer tarsus	effect size d = 1.60 minor - magnitude score of1 (co-varying with wing length)	0
Acoustic characters	has high total number of notes (n= 4)	has less total number of notes (n=2.9)	effect size d = -1.35 moderate - magnitude score of 2	2
	high minimum frequency in the song (f= 2082.8 Hz)	low minimum frequency in the song (f= 1106.7 Hz)	effect size d = -6.83 major - magnitude score of 3	3

Plumage characters and bare body parts	dull brown cap of breeding males	rufous cap of breeding males	different colour and tone of strongly demarcated body part major - magnitude score of 3	3
	dull brown (washed) upperparts	dull dark brown upperparts	different tone of significant area of feathering moderate - magnitude score of 1	1
	Showing a marked difference in moulting strategy		partial moulting into non-breeding plumage before breeding and arrest of moulting in breeding season. Moderate- magnitude score 2	2
	Discontinuous eye-lore of male in breeding plumage	Continuous eye-lore of male in breeding plumage	weak divergence in a plumage feature minor - magnitude score of 1	1
Ecological and behavioral characters	on average inhabits softer mud and dry grasslands	on average inhabits softer mud along tidal channels (Rheindt et al. 2011)	No magnitude score	0

Geographical characters	Resident subspecies in Sri Lanka and South-east India	Migration during winter, no overlapping breeding ranges with *C. a. seebohmi*	Parapatric geographic distribution - moderate - magnitude score - 2	2
Total assigned value				16

TABLE 3

Genetic divergence among *C. a. seebohmi, C. a. alexandrinus*, and *C. dealbatus*. Above diagonal: pairwise F_{ST} between groups. Below diagonal: estimates of evolutionary divergence over sequence pairs between groups. *C. nivosus* is also considered for the divergence estimates (P < 0.01 in all the analysis).

	C. seebohmi	*C. dealbatus*	*C. alexandrinus*
C. seebohmi		0.095	0.078
C. dealbatus	0.020574		0.013
C. alexandrinus	0.019909	0.001995	
C. nivosus	0.066708	0.048628	0.050291

FIGURE 1

Adult male (A) and female (B) *C. a. seebohmi* from Mannar Island Sri Lanka and adult male (C) and female (D) *C. a. alexandrinus* from Bohai Bay, China. Open wings of (E) *C. seebohmi* and (F) C. *alexandrinus*. Photo credits: (A-B, E: JJN, C-D: PQ, F: Chenjing Huang)

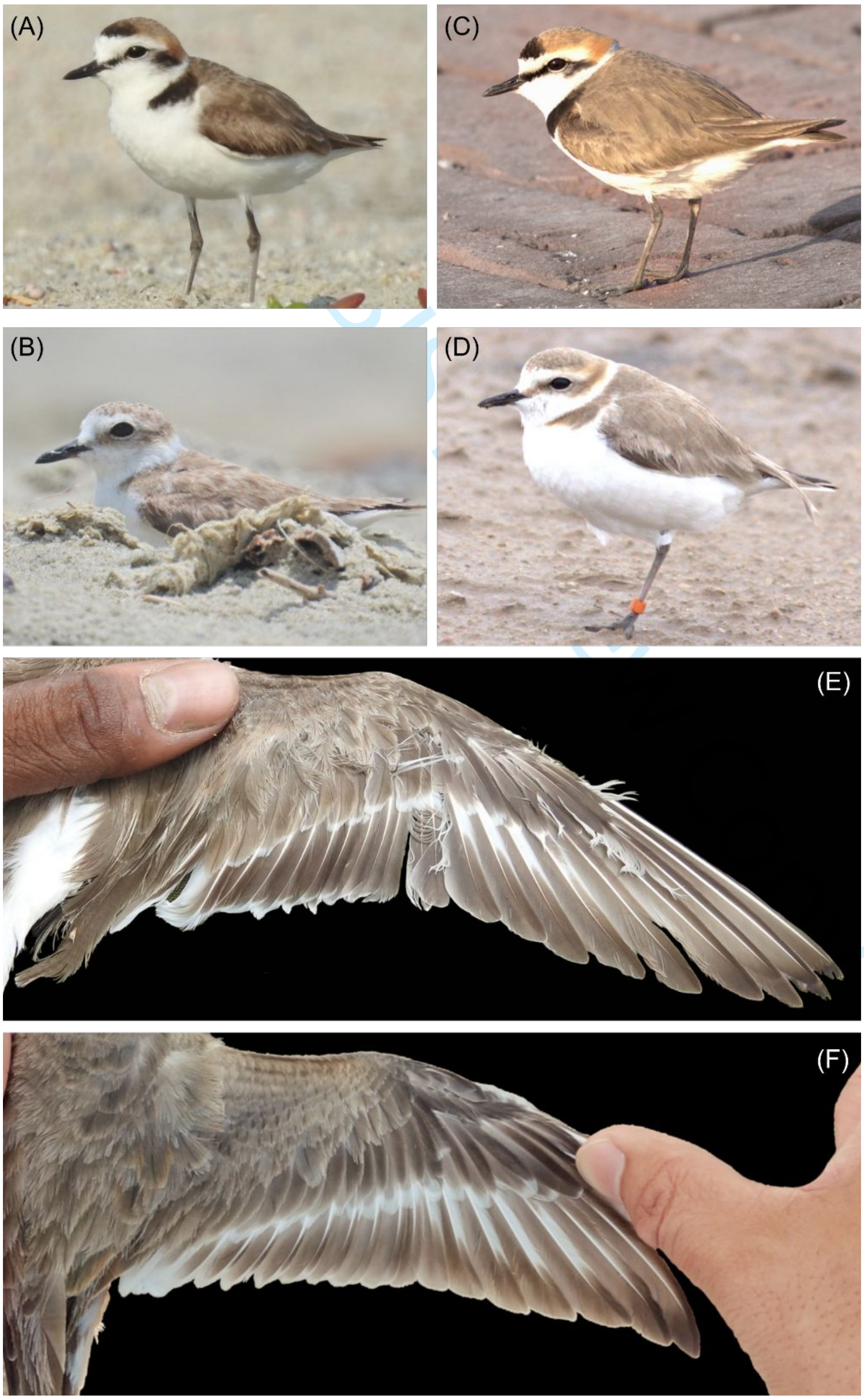


FIGURE 2

Map of Asia showing breeding range of *C. a. seebohmi*, *C. a. alexandrinus and C. dealbatus.* Inset (box) showing field sites in Sri Lanka (A) and China (B). Structure plots of Bayesian genetic clustering for three subspecies; K=4 (above) and K=3 (below) with respect to their sampling locations. Numbers represent: 1 Mannar and 2 Hambantota (Sri Lanka), 3-13 *C. a. alexandrinus* populations and 14-21 *C. dealbatus* populations (China), a-c locations of museum specimens at NMSL.

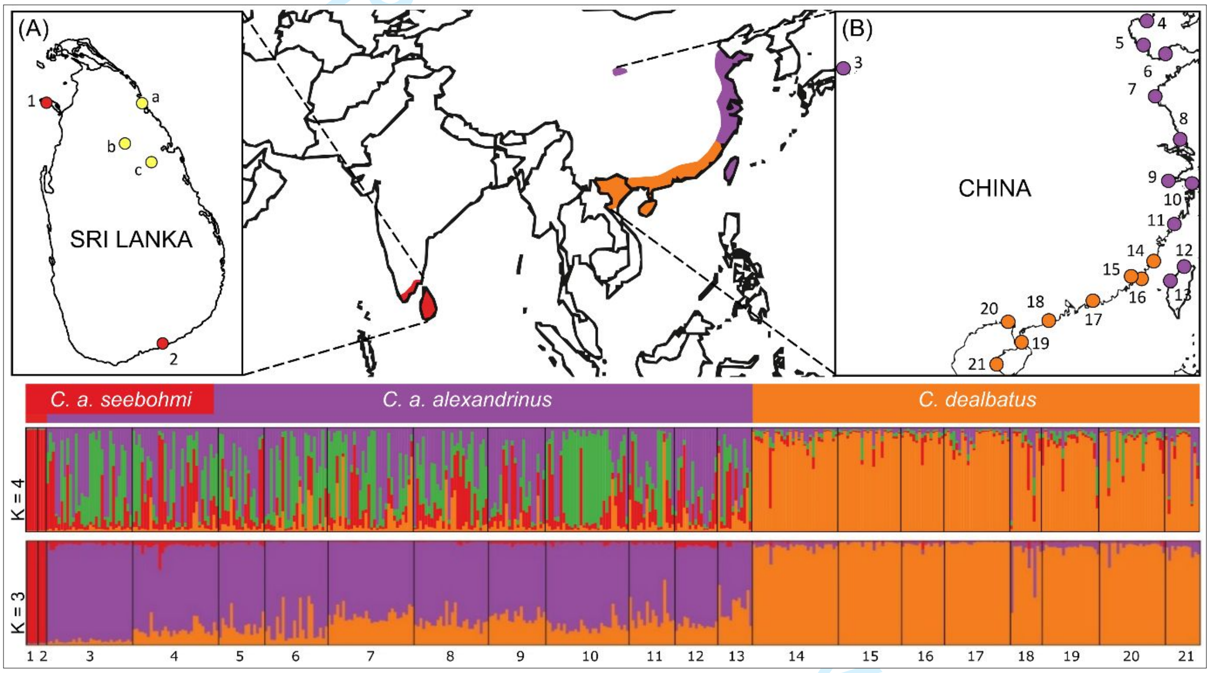
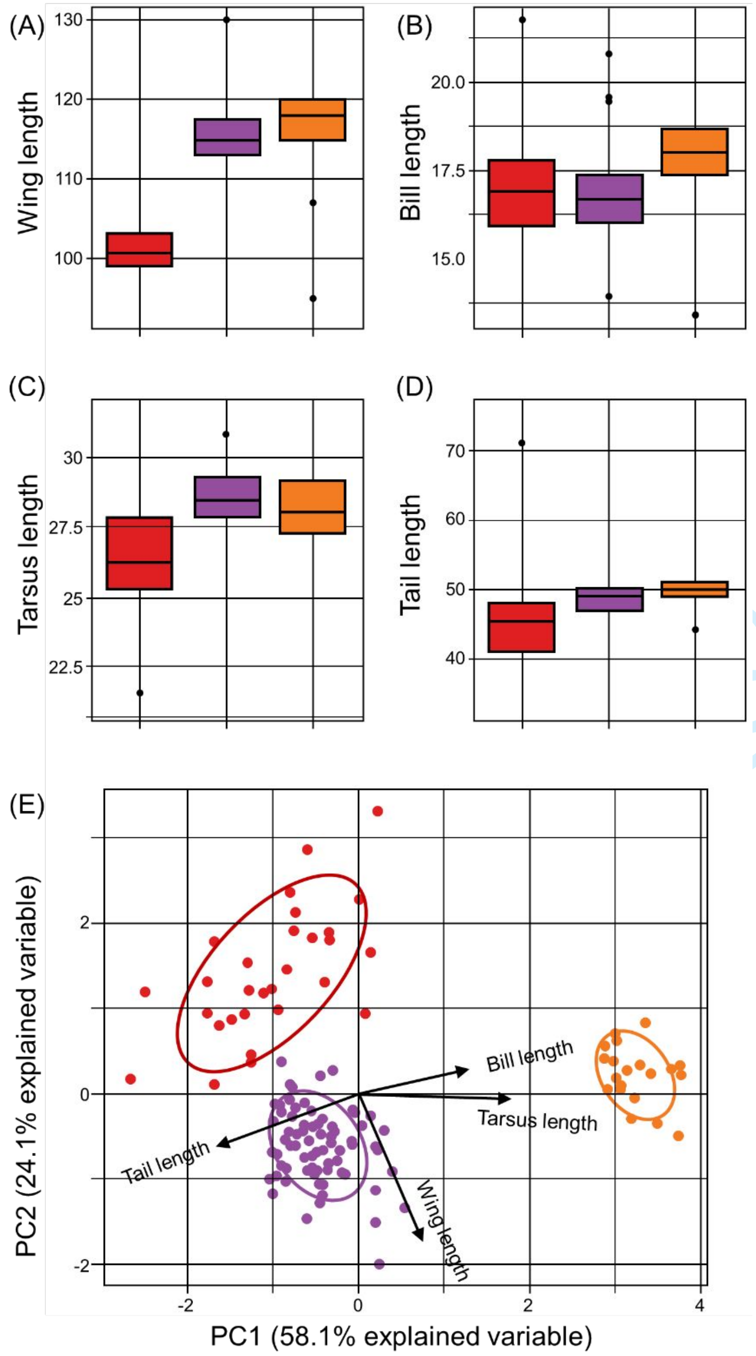


FIGURE 3

Morphometric analysis of *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus* indicated in: (A) wing length, (B) bill length, (C) tail length and (D) tarsus length of both male and female *C. a. seebohmi*, *C. a. alexandrinus* and *C. dealbatus*; horizontal lines within boxes represent the median values. The Principal Component Analysis (E) of *C.a. seebohmi, C.a. alexandrinus, and C. daelbatus.*

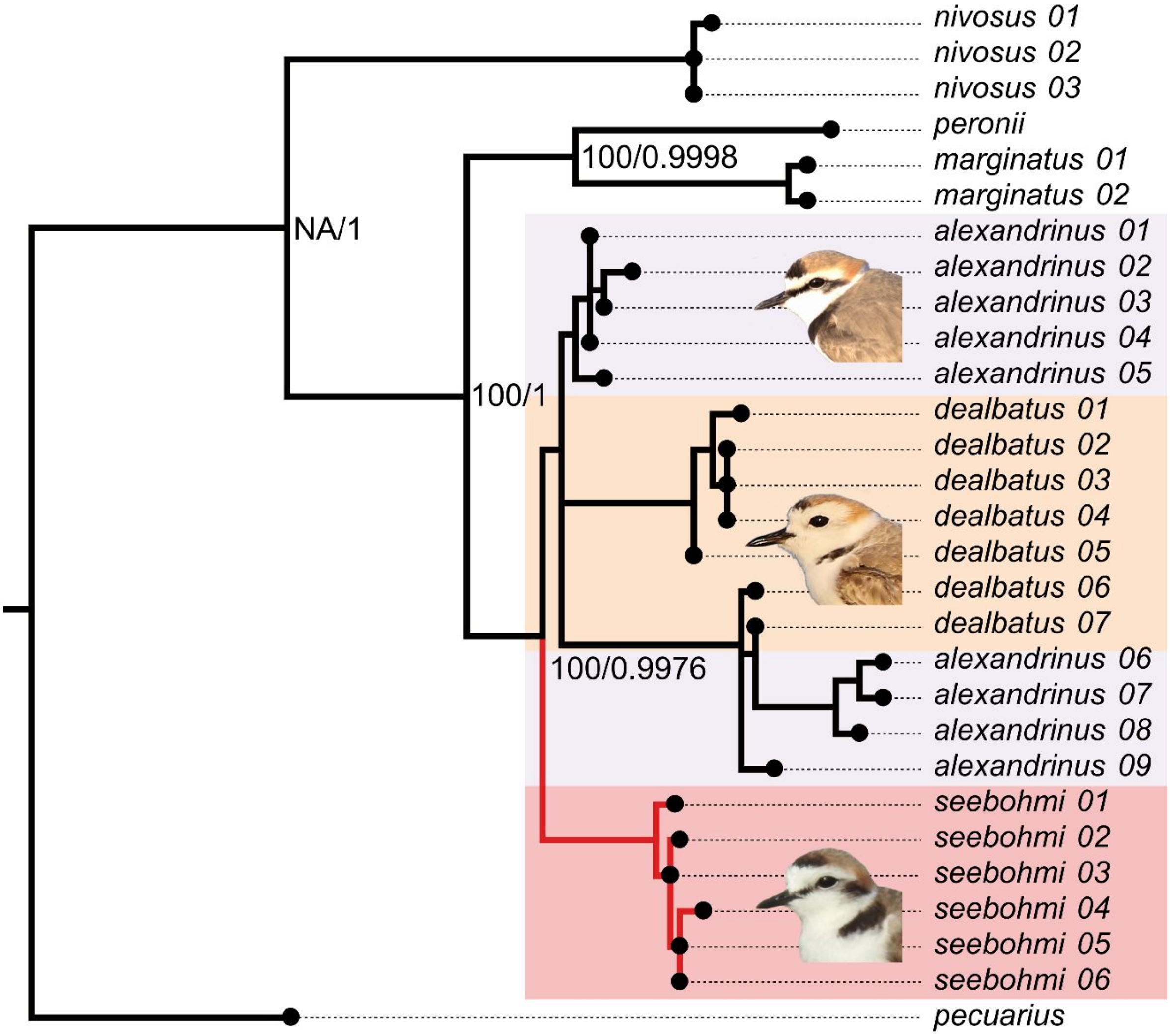


1055 **FIGURE 4**

1056 Phylogenetic affinities in the *C. alexandrinus* complex generated using mitochondrial CR and

1057 ND3 regions. Values above the branches indicate bootstrap values in Maximum likelihood

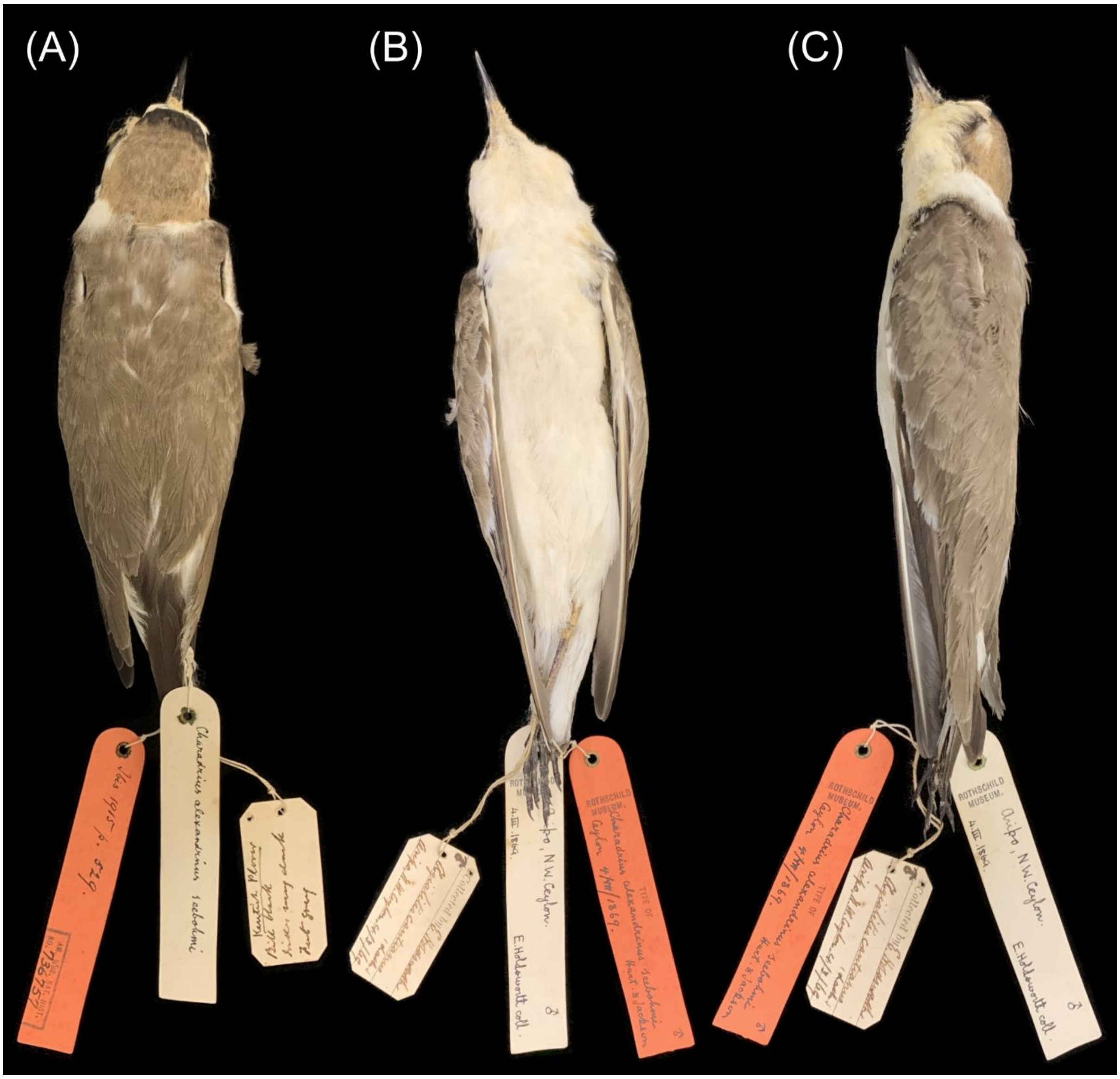
1058 framework, values below indicate posterior probability in Bayesian framework.

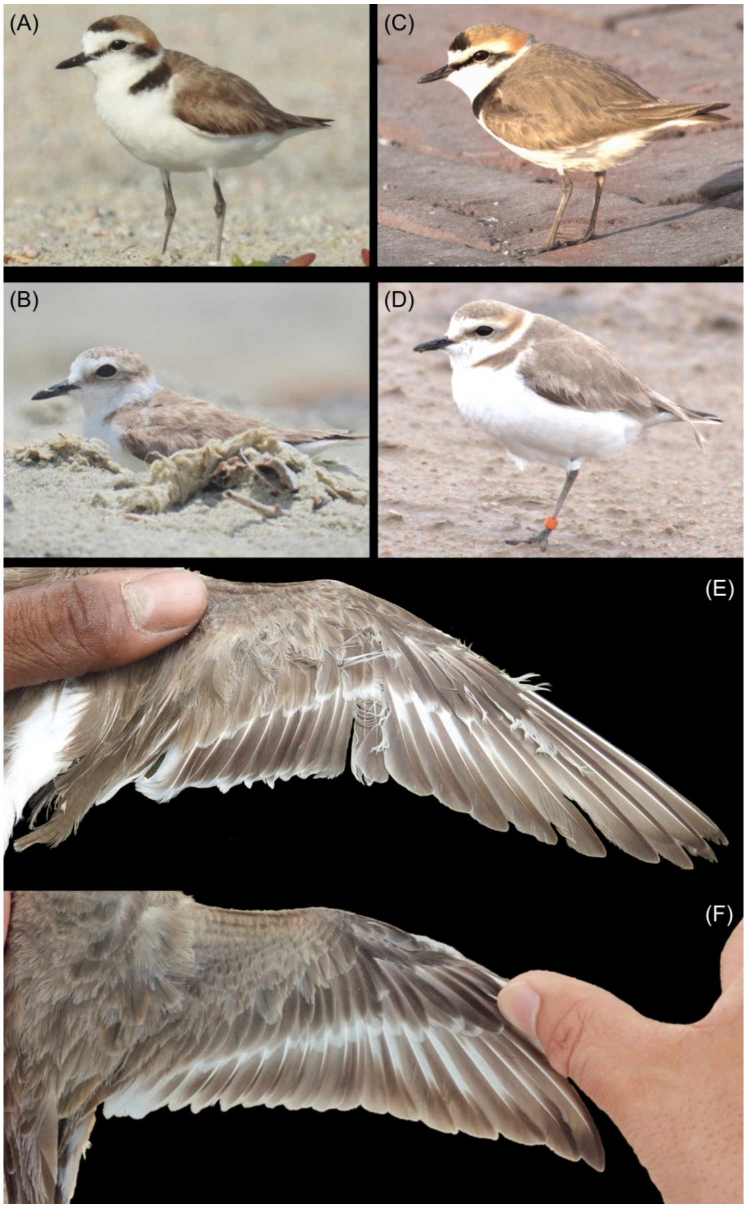


1059

FIGURE 5

Holotype: AMNH 736757: a male *C. a. seebohmi*, collected in 1872 from Arripu (near Mannar), Sri Lanka by E. W. H. Holdsworth. Dorsal (A), ventral (B) & side aspects (C). (Photo credit Paul R Sweet: American Museum of Natural History).





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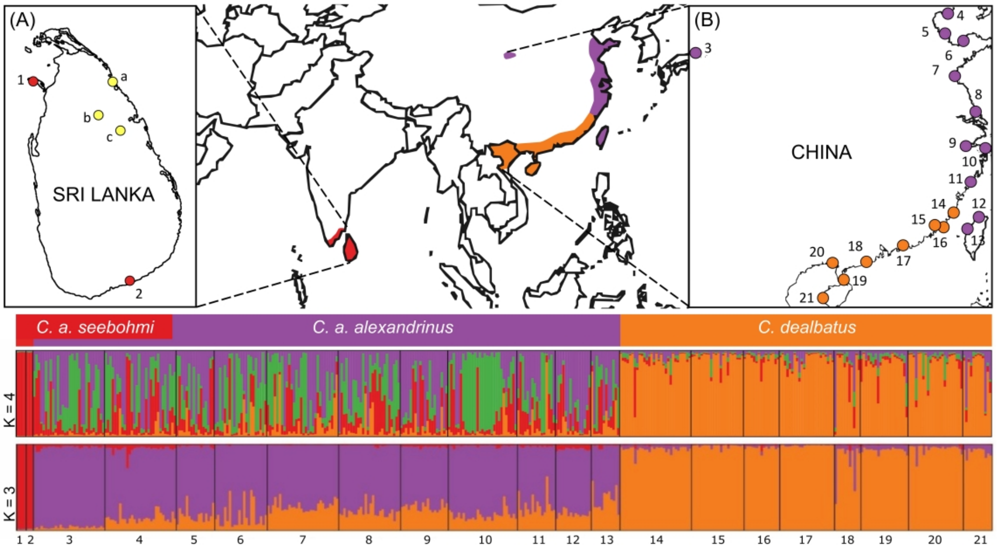

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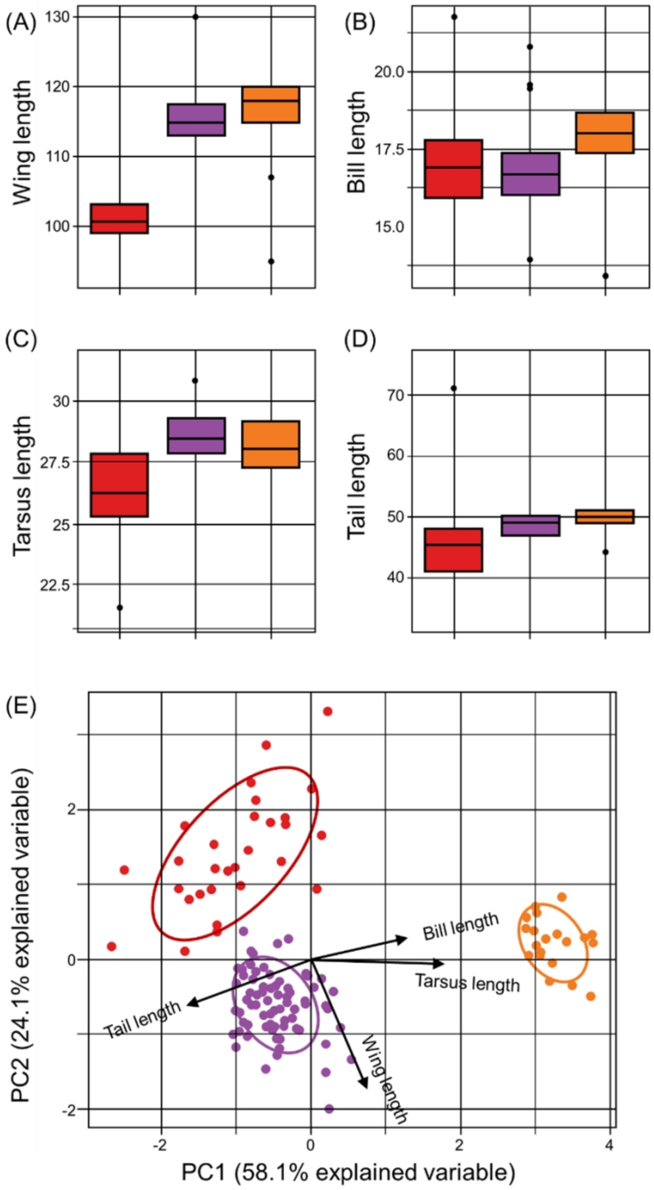

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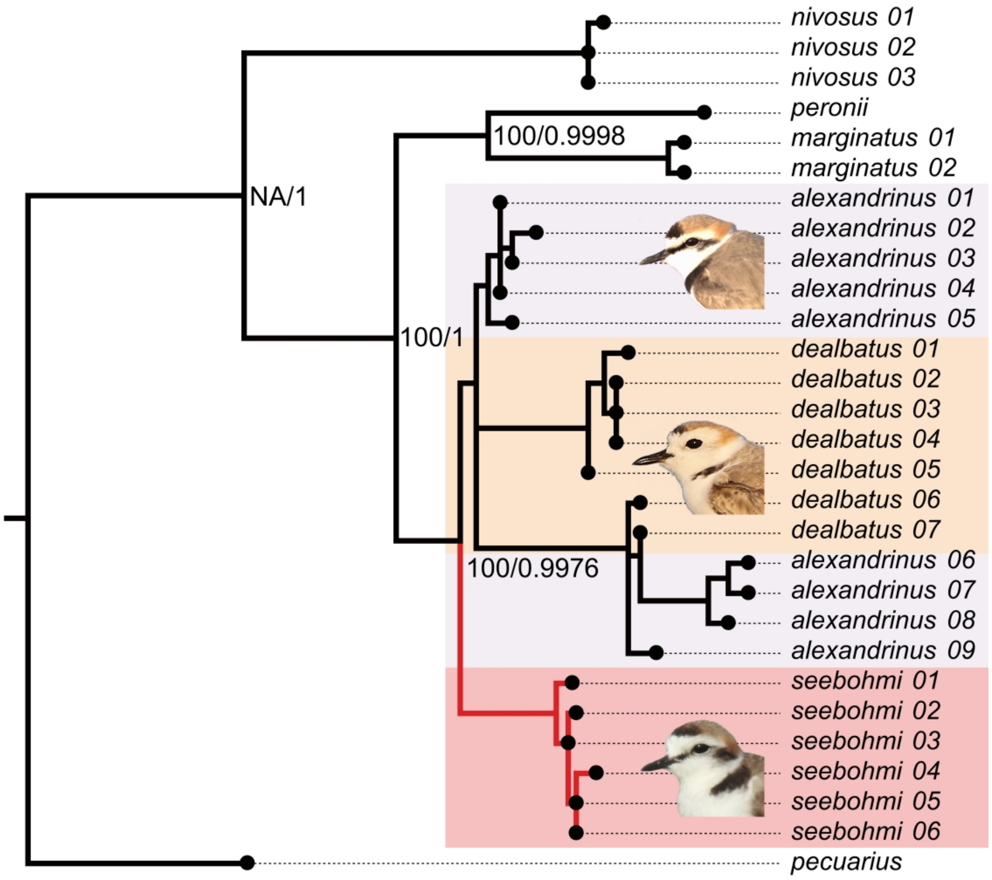

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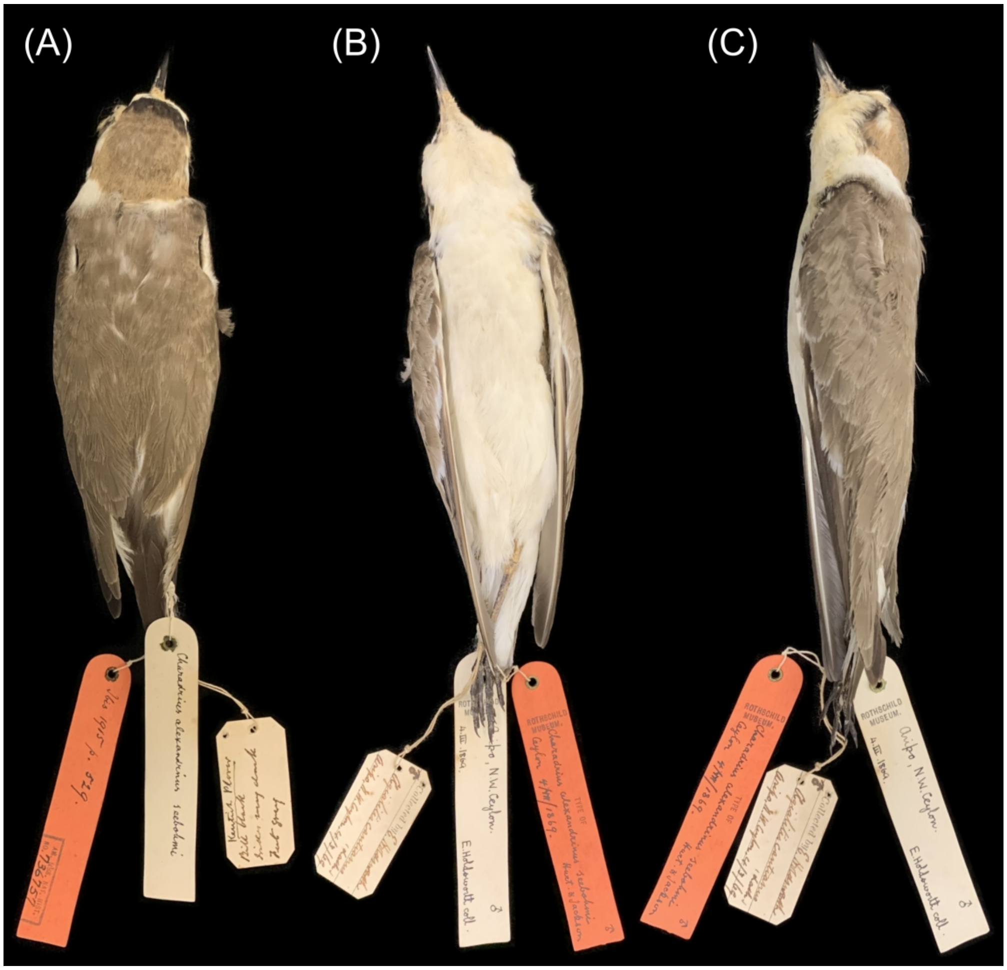

529x509mm (197 x 197 DPI)

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February 23, 2022

The Editor
IBIS,

Dear Sir,
Herewith, we submit our manuscript, 'Re-evaluation of the taxonomic status of the 'diminutive' Kentish Plover *Charadrius alexandrinus seebohmi* based on phenotypic and genetic analyses' for publication as an original article.

The Kentish Plover is a common shorebird of Eurasia and North Africa and is an emerging model species of breeding system evolution. Here, we focussed on *C. a. seebohmi* (Hartert & Jackson 1915), a resident population found in the southern tip of the Indian Subcontinent, and re-evaluated its taxonomic status based on an assortment of phenotypic, and genetic characteristics. We showed that *C. a. seebohmi* has different body size, moulting pattern and plumage colouration from its conspecifics. Based on two mitochondrial, two sex-linked and 11 autosomal microsatellite markers, we showed that it is sister to the clade of Kentish and White-faced Plovers with an estimated 1.34-million-year divergence. We examined ornithological records of major museum collections of *C. a. seebohmi* in Asia, Europe and North America. Based on the genetic, phenotypic, allochronic migratory pattern and breeding range, we propose species status for *C. a. seebohmi* as *Charadrius seebohmi*.

We believe that this study provides an illustrative example of divergence in isolation, and the use of modern and historic material for species delimitation in ornithology.

Our submission consisted of the complete text including five Figures and three Tables, and Supplementary Material. Please note that this manuscript has not been submitted for publication elsewhere and it does not overlap with previously published work. All contributors have read and approved the content.

We thank you in advance for your consideration of this manuscript for publication.

Sincerely,

Sampath Seneviratne Ph.D
Corresponding author on behalf of Yang Liu, Jude Niroshan, Jonathan Martinez, Pinjia Que, Chentao Wei, Sanjaya Weerakkody, Gayomini Panagoda, Jagathpriya Weerasena, Tamás Székely and Alex Bond