

Population structure of *Calidris alpina* and separation of subspecies at a stopover on the intertidal mudflats of Jiangsu Province, China

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Abstract

Dunlin (*Calidris alpina*) is a polymorphic species with a complex of subspecies. A migration stopover site on the intertidal mudflats of Jiangsu Province, China, has a pivotal role in the migratory connectivity of dunlin along the East Asian–Australasian Flyway (EAAF). However, to date, the dunlin subspecies that visit the coast of China during migration remains uncertain. To determine the subspecies, an integrated approach based on mitochondrial DNA, ring recoveries, and morphological traits was used to analyze dunlins sampled at the Jiangsu stopover site. Alaskan and Beringian lineages were the two dominant lineages that migrated through Jiangsu, and the number of dunlins from the Alaskan lineage greatly exceeded that from other lineages. According to genetic analysis, the proportion of identified lineages was greater than 70%. At least four subspecies were detected in eastern Jiangsu Province, including *C. a. actites*, *C. a. kistchinski*, *C. a. sakhalina*, and *C. a. arctica*. There were significant differences in morphological characters between years, suggesting that proportions of subspecies at the stopover varied over time and that different subspecies adopted different migratory strategies and timings. The findings of this study highlight the need to further consider how subspecies contribute to the composition of populations and migratory connectivity of dunlin.

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Dunlin (*Calidris alpina*) is a polymorphic species with a complex of subspecies. A migration stopover site on the intertidal mudflats of Jiangsu Province, China, has a pivotal role in the migratory connectivity of dunlin along the East Asian–Australasian Flyway (EAAF). However, to date, the dunlin subspecies that visit the coast of China during migration remains uncertain. To determine the subspecies, an integrated approach based on mitochondrial DNA, ring recoveries, and morphological traits was used to analyze dunlins sampled at the Jiangsu stopover site. Alaskan and Beringian lineages were the two dominant lineages that migrated through Jiangsu, and the number of dunlins from the Alaskan lineage greatly exceeded that from other lineages. According to genetic analysis, the proportion of identified lineages was greater than 70%. At least four subspecies were detected in eastern Jiangsu Province, including *C. a. actites*, *C. a. kistchinski*, *C. a. sakhalina*, and *C. a. arctica*. There were significant differences in morphological characters between years, suggesting that proportions of subspecies at the stopover varied over time and that different subspecies adopted different migratory strategies and timings. The findings of this study highlight the need to further consider how subspecies contribute to the composition of populations and migratory connectivity of dunlin.

Keywords: *Calidris alpina*, Subspecies, Stopover, Population structure

Introduction

Migratory shorebirds with long-distance migration and complex phylogeny are an essential component of global biodiversity and include the endangered Spoon-billed sandpiper and Nordmann's greenshank (Shumway et al. 2022). Recently, the migratory routes of many shorebirds, such as *Phalaropus lobatus* and *Calidris tenuirostris*, have been described by satellite or other techniques, which indicate that mazy migratory networks are formed by different species or subspecies (Gill et al. 2009, Lisovski et al. 2016, Mu et al. 2018). However, with rapid declines in populations of shorebirds due to diverse environmental threats, effective conservation requires improved understanding of the migration strategies and population structures of subspecies in key stopover sites (Warnock 2010, Sutherland et al. 2012, Melville et al. 2016, Studds et al. 2017, Ma et al. 2022). Identification of subspecies is crucial to determine where and when special subspecies populations are migrating (Boulet and Norris 2006, Gill et al. 2013).

Stopover sites are essential components of the flyways of migratory bird populations that link breeding sites with nonbreeding areas (Anderson et al. 2019, Wang et al. 2022). Migration stopover sites are vital in the whole migratory process by providing large quantities of food to help mixed populations of species or subspecies replenish energy reserves for continued migration (Moore and Simons 1990, Dunn 2001). The region of the southern Yellow Sea in Jiangsu Province, China, is important for migrating shorebirds, and many vulnerable shorebirds preferentially select the area as a refueling stop (Chen et al 2015, Ma et al. 2013, Tong et al. 2012). Overall, ring recoveries, geolocators, and morphological comparisons indicate that at least four subspecies of the dunlin *Calidris alpina* migrate along the East Asian–Australasian Flyway (EAAF), including *C. a. arctica*, *C. a. sakhalina*, *C. a. kistchinski*, and *C. a. actites* (Lagassé et al. 2022). Moreover, two additional dunlin subspecies, *C. a. alpina* and *C. a. centralis*, may stage at the Jiangsu stopover.

Morphological, genetic, ringing, and leg-flagging methods are often used to determine migratory connectivity and population structure (Verkuil et al. 2012, Weston et al. 2020). Ring recoveries, recaptures, and resightings are valuable in providing accurate records of the links between breeding, stopover, and wintering sites for dunlin (Evans 1984, Gill et al. 2013, Lagassé et al. 2020). Studies of genetic and morphological variation also provide an outline of the phylogeography and migratory patterns (Greenwood 1984, Wennerberg et al. 1999, Popovic et al. 2019). Morphological characters, especially bill, wing, and tarsus lengths, have also been used to identify the origins of dunlins from Alaska and the Russian Far East region (Greenwood 1986), but sexual dimorphism in size requires that birds be sexed for accurate analysis of morphological data. Analyses of dunlin mitochondrial DNA (mtDNA) can indicate breeding origins and migratory patterns of birds from different breeding sites as well as genomic homogeneity among overwintering populations (Wenink et al. 1996, Wennerberg 2001, Popovic et al. 2019).

The dunlin is a typical example of a polytypic wader with an extensive distribution for which analysis of phylogeographic patterns (Marthinsen et al. 2007) is suitable to show spatial and temporal patterns at a subspecies level. Five lineages of dunlin may occur in the Palearctic region, namely the European, Siberian, Beringian, Alaskan, and Canadian lineages (Wenink and Tilanus 1996). Moreover, in reviews of the systematics, dunlin is divided into 10 subspecies (Greenwood 1986). Dunlin subspecies from various breeding populations use different Palearctic migration flyways (Gromadzka 1989) and exhibit regional segregation and high site fidelity along a flyway (Lagassé et al. 2020). For example, two subspecies of dunlin, *C. a. arctica* and *C. a. sakhalina*, are dominant in different regions along the EAAF (Lagassé et al. 2022).

Approximately 570,000 dunlins are estimated to use the EAAF to migrate from breeding to wintering areas (Jing-Gong et al. 2009). Although the migratory routes of different dunlin subspecies have been described (Lagassé et al. 2020), it is difficult to determine which subspecies of dunlin stopover at special key sites, which hinders understanding of subspecies migration. How many dunlin subspecies are present during key migratory periods is uncertain, and additional detailed evidence is needed to detect migratory origins and population composition of dunlins at key stopover sites (Webster et al. 2002). Coastal mudflats in southern Jiangsu Province are always important for mixed populations of different dunlin subspecies migrating along the EAAF. The aim of this study was to identify the subspecies of dunlin and their proportions using the stopover site in Jiangsu Province. Of particular concern was *C. alpina arctica*, which is considered

a priority species for the Arctic Migratory Birds Initiative conservation efforts (Weiser et al. 2018). To analyze the subspecies of dunlin migrating on the EAAF, an integrated approach was used that combined morphometry, ringing recovery, and genetic phylogeny methods.

Methods

Study area

The mudflats in Jiangsu Province support large and diverse and flocks of migratory shorebirds, including many rare and endangered species, and are crucial to global biodiversity conservation. The sampling sites Tiaozini (TZN) and Yongkou (YK) were areas of intertidal mudflats separated by 60 km (Fig. 1). In 2019, Tiaozini was selected as a Natural World Heritage site as the largest area of intertidal mudflats in the world. The mudflats have a pivotal role in the migrations of shorebirds and are key wintering, breeding, and staging sites on the EAAF. At least four hundred species and 50 million migratory birds are recorded in the study area, which is listed under Ramsar wetlands of international importance (Yang et al. 2017).

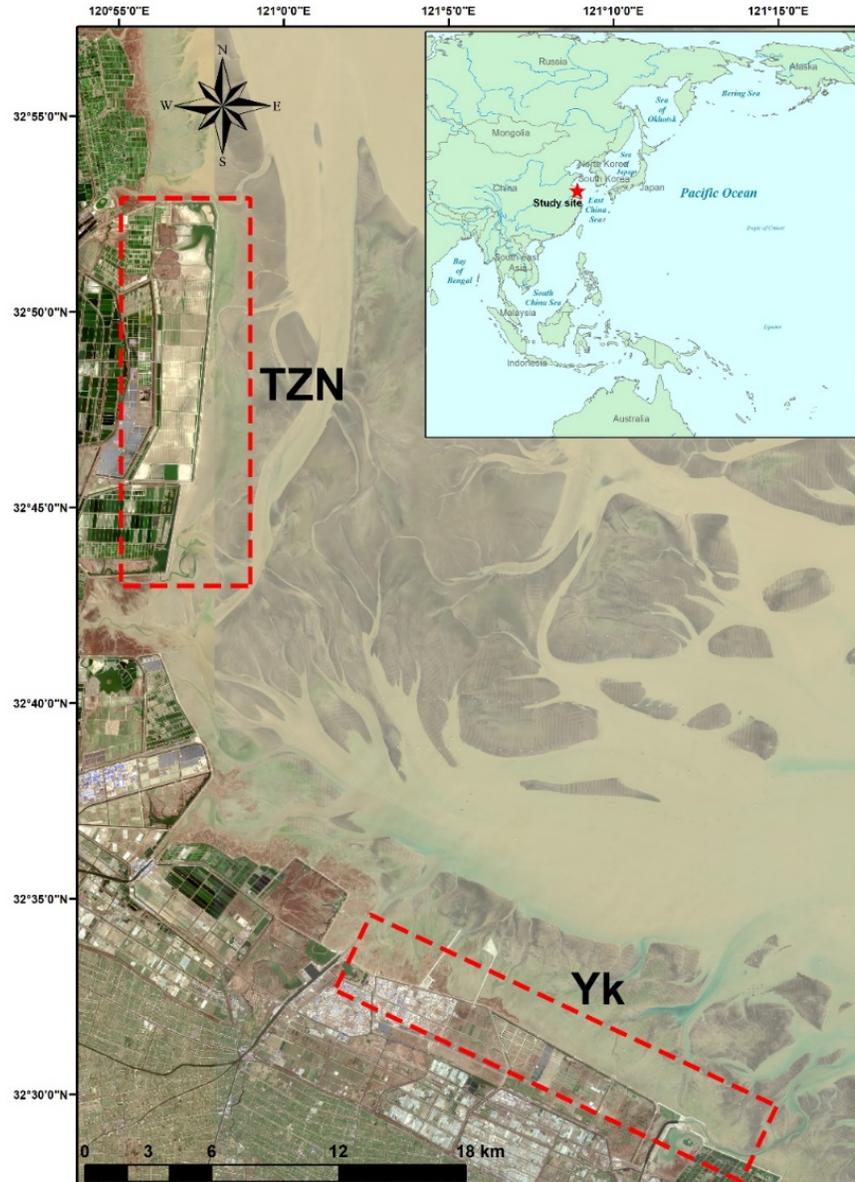


Figure 1 Map of sampling locations for dunlin on the coast of Jiangsu Province, China. Tiaozini (TZN); Yangkou (YK). Dashed red frames represent the main bird-banding areas.

Capture methods and sampling

Dunlin individuals were captured during the autumn migration period in 2015 (12–20 September) and 2016 (26 September to 6 October). Birds were captured on intertidal flats and adjacent lagoons behind the sea wall where they roosted at high tide in different ways, depending on time of day, weather conditions, and stage of the tidal cycle. Canon nets and elastic-powered whoosh nets were used to trap birds in daylight, whereas mist nets and dazzling with lights were used at night. A total of 791 dunlin individuals were captured. Birds were placed in keeping cages before being processed. Birds were banded with a metal band and color flag (upper green and lower blue). Maximum chord wing length was measured to the nearest 1.0 mm with a wing rule, and bill and total head lengths were measured to the nearest 0.1 mm with dial calipers. Blood samples,

20–50 μL , were collected from some individuals by venipuncture from the wing vein and stored in 0.001 M Ethylene Diamine Tetraacetic Acid (EDTA). Cotton swabs were used in beaks to collect epithelial cells, and coverts from the belly were also collected. All specimens were stored in 75% ethanol and immediately placed into an icebox.

Ring recoveries and resightings

As many ring recoveries and resightings of dunlins as possible were complied. In addition to the recaptures and resightings from Jiangsu in 2015 and 2016, information on ring recoveries and resightings was obtained from other sites by organizations and social volunteers. The spatial distribution map of the recoveries was drawn in Arcgis 10.2.

DNA studies

DNA was extracted from blood, epithelial cells, and calamus of feathers by a phenol–chloroform method according to standard protocol (Sambrook et al. 1989, Smith and Schantz 1996). Two fragments of mtDNA were amplified by PCR. The control region (CR) of mtDNA was amplified using the primers L98 and H772 (Wenink et al. 1993). In addition, the primers L13706 and H14562 (L13706, 5'-AATGGCCCCAAACCTCCGAAAAT; H14562, 5'-GGATTGAGCGTAGGATGGCGTAT) were designed to amplify the cytochrome *b* (*Cytb*) gene. The CR and *Cyt b* segments were both amplified for 35 cycles, using the following temperature profile: denaturation at 94°C for 30 s, annealing at 50.9°C (CR) / 50°C (*Cytb*) for 30 s, and a final extension at 72°C for 1.5 min. The PCR amplifications were conducted in a 25- μL reaction system containing 1.0 μL of DNA template (100 ng/ μL), 1.0 μL of each primer (10 μM), 3.0 μL of 10 \times buffer, 2.0 μL of MgCl_2 (25 mmol/l), 2.0 μL of dNTPs (2 mmol/l), 1.0 μL of Taq DNA polymerase (1U; Takara, Japan), and 15 μL of sterilized double-distilled water. After checking the PCR amplifications by electrophoresis, PCR products were sequenced in two directions with each primer on an ABI 3731XL sequencer.

The sex of individuals was identified by a molecular genetic sexing method. After DNA was extracted, the primers P2 and P8 were used in PCR to amplify the sex chromosomes (Griffiths et al. 1998). Then, the sex of individuals was assigned by reading the numbers of bands separated by electrophoresis in a 2% agarose gel.

Phylogenetic analysis

A 633-bp sequence was obtained from the *Cyt b* gene and a 478-bp sequence was obtained from the CR, which were integrated into one 1111-bp sequence. After excluding some samples of poor quality, 78 individual sequences from 2015 and 173 individual sequences from 2016 were obtained. For all mtDNA sequences, which combined the *Cyt b* and CR genes, coalescent sequences were aligned by the algorithm Clustal W in Mega v7 (Kumar et al. 2016). Then, DnaSP v5 and FaBox websites were used to generate haplotypes from the original individual sequences (Villesen 2007, Librado and Rozas 2009). In addition, 94 reported haplotypes were also downloaded from the National Center for Biotechnology Information (NCBI) for further alignment. The haplotypes were derived from 370 dunlin individuals breeding in eastern Asia and Alaska, including six subspecies, *C. a. actites*, *C. a. kistchinski*, *C. a. sakhalina*, *C. a. hudsonia*, *C. a. pacifica*, and *C. a. arctica* (GenBank accessions for CR: KP205084–KP205177; GenBank accessions for *Cytb*: KP205178–KP205271) (Miller et al. 2014).

To infer to the phylogenetic relationships between dunlins from the breeding areas and those sampled at Jiangsu, all sequences were analyzed with Bayes inference (BI) and maximum-likelihood (ML) algorithms. The program PhyML v3.1 was applied to infer phylogenetic relationships among haplotypes under the ML criterion, with 1000 bootstrap replicates used to evaluate clade support (Guindon et al. 2010). The best-fit nucleotide substitution model for ML analysis was distinguished using Jmodeltest v2.1.7 based on the Akaike information criterion (AIC) (Darriba et al. 2012). MrBayes v3.2.2 was used to infer phylogenetic relationships by a multigene Bayesian algorithm based on the model, as suggested by Mrmodeltest v3.7 software (Ronquist et al. 2012). Four concurrent Markov Chain Monte Carlo chains (one cold chain and three hot chains) were

run simultaneously for 5.0×10^7 generations. Trees were sampled every 1000 generations, and the burn-in of the initial 25% of samples was discarded. Tracer v1.5 was used to test and check the reliability of the results of Bayesian analysis.

Statistics

Univariate comparisons of bill and wing lengths between sexes and years were implemented. To determine differences in phylogenetic relationships and morphological features between years, it was assumed the composition of different lineages might vary between the 2 years. The morphological data set of dunlins from lineages inferred from phylogenetic relationships was analyzed further. Bill, wing, and total head lengths among different lineages and sexes were compared. The fit to the normal distribution and homogeneity of variance of morphological data were tested, and *t*-tests or Mann–Whitney U-tests were performed sequentially. All statistical analyses were performed with SPSS 22.0.

Results

Ring recovery

Migratory connections based on ring recoveries and leg-flag sightings in eastern Jiangsu are presented in Table 1. Some dunlin individuals staging in Jiangsu came from Chukchi and Kamchatka peninsulas, Sakhalin Island, Yalujiang Estuary, South Korea, Chongming Island, Taiwan, Hong Kong, Beibu Gulf, and Leizhou Bay. The ring recoveries produced a rough migratory map that showed dunlin breeding in the Far East of Russia migrated through the Yalujiang Estuary, Jiangsu, and Chongming Island to winter in southern China (Supporting information Fig. S1). The numbers of ring recoveries from Kamchatka Peninsula and Sakhalin Island were greater than those from other locations. However, there were no resightings that indicated dunlins migrated from breeding areas in Alaska or the west of Russia.

Table 1. Summary of ring recoveries in the study area and associated areas

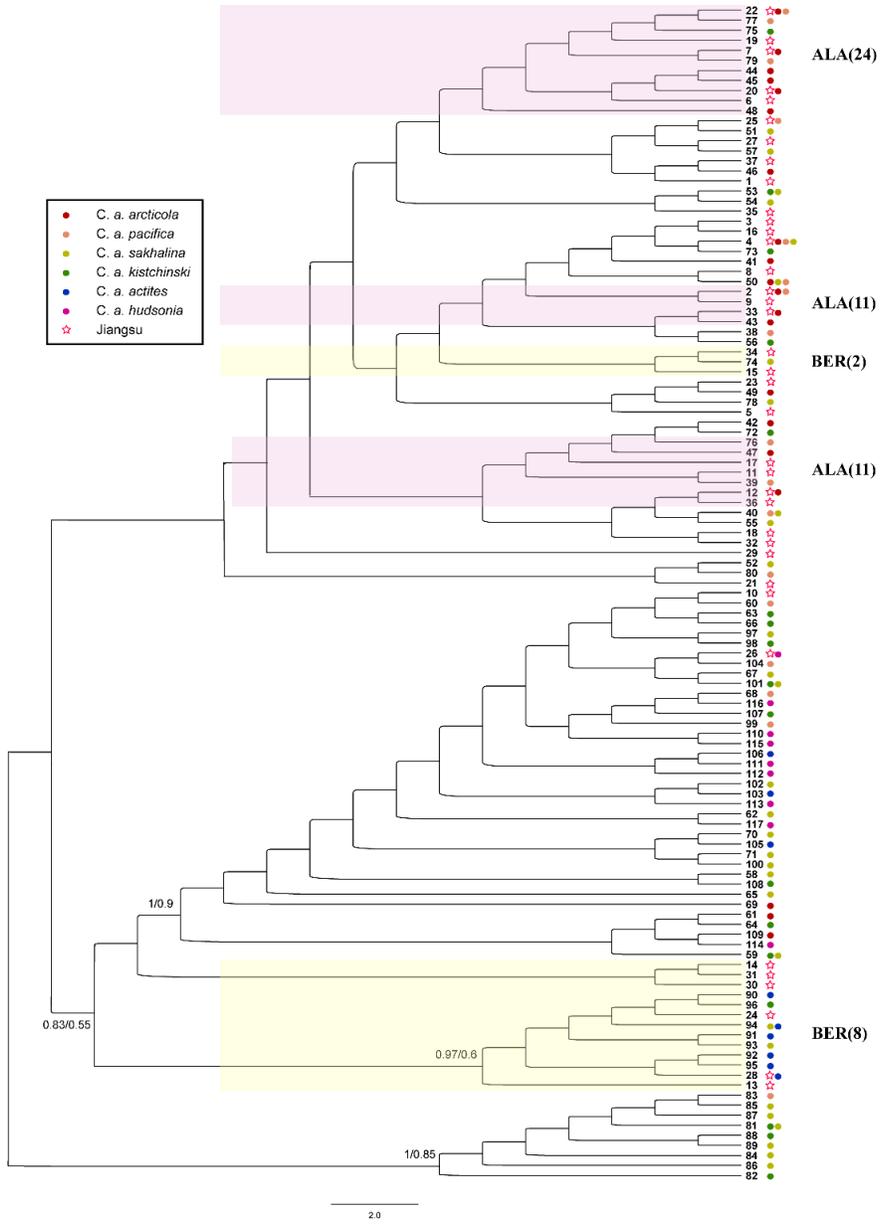
No.	Area of ringing	Area of recovery	Number of recoveries
1	Chukchi Peninsula	Jiangsu	1
2	Kamchatka Peninsula	Jiangsu	14
3	Sakhalin Island	Jiangsu	9
4	Yalujiang Estuary	Jiangsu	1
5	Jiangsu	South Korea	2
6	Chongming Island	Jiangsu	5
7	Jiangsu	Taiwan	5
8	Hong Kong	Jiangsu	1
9	Jiangsu	Beibu Gulf	1
10	Jiangsu	Leizhou Bay	1

Phylogenetic analysis

To analyze mtDNA sequences, the concatenated 1111-bp Cyt *b* and CR sequence data from Jiangsu were aligned with NCBI sequences. One hundred and seventeen unique haplotypes were identified from the data set combining NCBI and 78 individuals, and 128 unique haplotypes were identified from the data set combining NCBI and 173 individuals. Then, 79 unique haplotypes were identified by collapsing the concatenated sequences from 2015 and 2016. For the 117 haplotypes, the best-fit model from Bayesian analysis was the GTR+I+G model. The software Jmodeltest v2.1.7 identified the TrN+I+G model as most appropriate from ML analyses. The GTR+I+G and TIM3+I+G models were identified as most appropriate from BI and ML analyses of 128 haplotypes. The HKY+I+G and TPM2uf+I+G models were suitable from BI and ML analyses of 79 haplotypes, respectively. The two trees were all paraphyletic and consensus was from an overall perspective with credible bootstrap values for all relationships among branches. Dunlins from a stopover in Jiangsu shared haplotypes or formed sister relationships with haplotypes from six subspecies,

including *C. a. actites* , *C. a. kistchinski* , *C. a. sakhalina* , *C. a. hudsonia* , *C. a. pacifica* , and *C. a. arctica* .

Haplotypes from Jiangsu and the breeding areas combined were clustered into two lineages, Alaskan and Beringian lineages, based on the percentage of speculated subspecies, which suggested that dunlins staging in Jiangsu migrated from Beringian and Alaskan areas. The corresponding numbers of individual dunlins are in parentheses in Fig. 2, and the numbers of dunlin individuals at the end of each branch are shown in Tables S1 and S2. Fifty-six individuals (78 in total) were inferred into the two lineages in 2015, and the proportion identified was 71.79%. One hundred and thirty-six individuals (173 in total) were inferred into two lineages in 2016, and the proportion identified was 78.61%. Twenty-two individuals in 2015 and 37 individuals in 2016 could not be identified. The proportion of Alaskan birds increased from 58.97% in 2015 to 63.58% in 2016, and the proportion of Beringian birds increased from 12.08% in 2015 to 15.02% in 2016. Simultaneously, the proportion of unknown birds decreased from 28.21% in 2015 to 21.39% in 2016. Three subspecies, *C. a. arctica* , *C. a. sakhalina* , and *C. a. kistchinski* , were more common in Jiangsu than other subspecies (Fig. 2). Moreover, distinct from the phylogeny shown in Fig. 2a, there were some haplotypes found only in Jiangsu that were assigned to a single clade of unknown subspecies in Fig. 2b. Notably, some of the dunlin individuals from Alaskan breeding areas detected at Jiangsu had a close phylogenetic relationship with *C. a. hudsonia*.



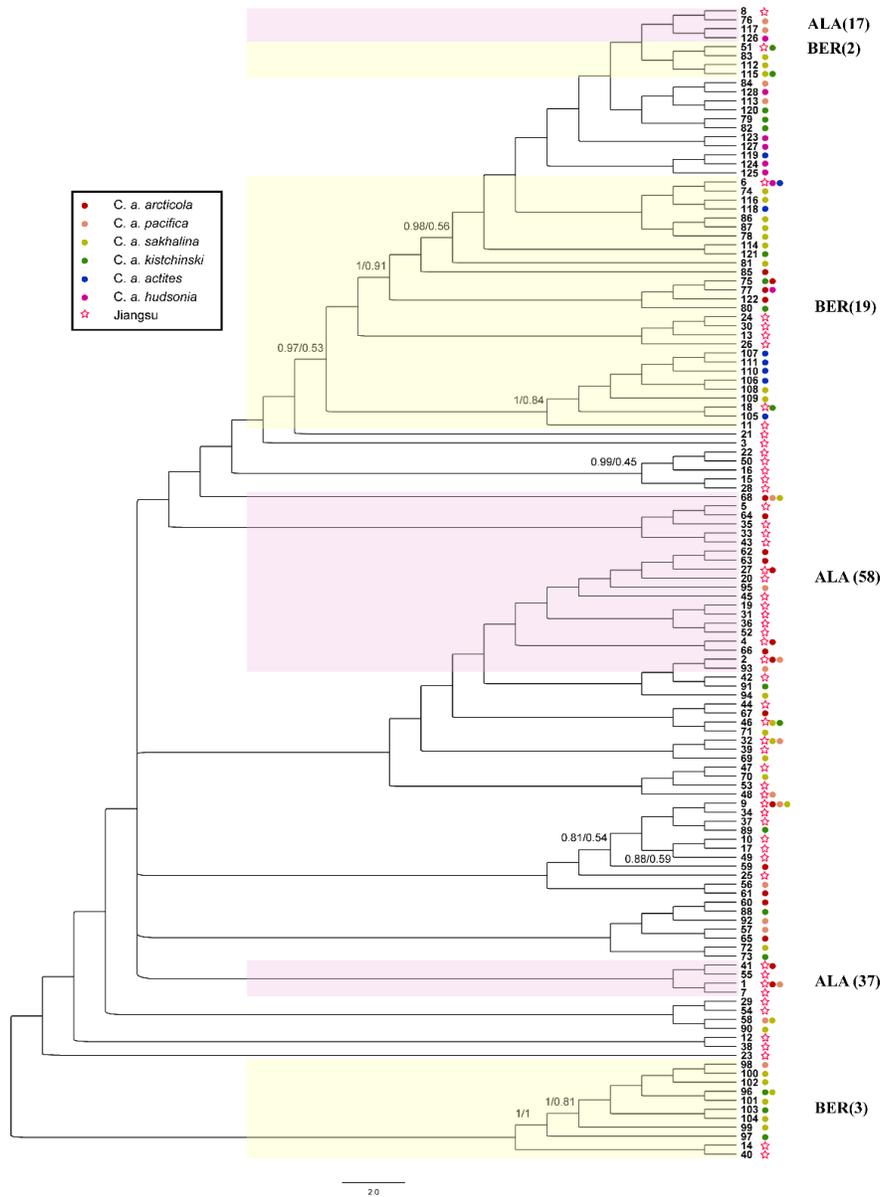


Figure 2 Bayesian and maximum likelihood (ML) trees for dunlin mitochondrial DNA haplotypes from Jiangsu in different years and from different breeding areas. (a) 2015 and (b) 2016. Numbers near nodes are posterior probabilities from Bayesian analysis and bootstrap values from ML analysis (BI/ML). Colored circles indicate subspecies of birds sampled in breeding areas or at Jiangsu (see key), which are grouped into Alaskan (ALA, pink shading) and Beringian (BER, yellow shading) lineages. The number of individuals from different lineages in different years is in parentheses.

Sixty-seven individuals were identified as males and 99 individuals were identified as females using a molecular method. The proportion of sexed birds decreased from 85.90% in 2015 to 57.23% in 2016. Owing to incomplete morphological data, the number of individuals was different in different morphological statistics. Scatter plots indicated little overlap in bill and wing lengths between the two years (Fig. 3). For males, wing length and bill length data conformed to a normal distribution, and both were significantly different between years (wing length, $t = 3.004$, $df = 44$, two-tailed $P = 0.004$; bill length, $t = 7.612$, $df = 44$, two-tailed

$P < 0.001$). Wing and bill lengths of males were shorter in 2015 than in 2016 (Fig. 3b). For females, wing length data conformed to a normal distribution, whereas bill length data did not fit a normal distribution. Only bill length was significantly different between the two years for females (wing length, $t = 1.657$, $df = 84$, two-tailed $P = 0.105$; Mann–Whitney U-test: bill length, $Z = -2.126$, $P = 0.033$). Female dunlins in 2015 tended to have longer bills and wings than those in 2016 (Fig. 3a).

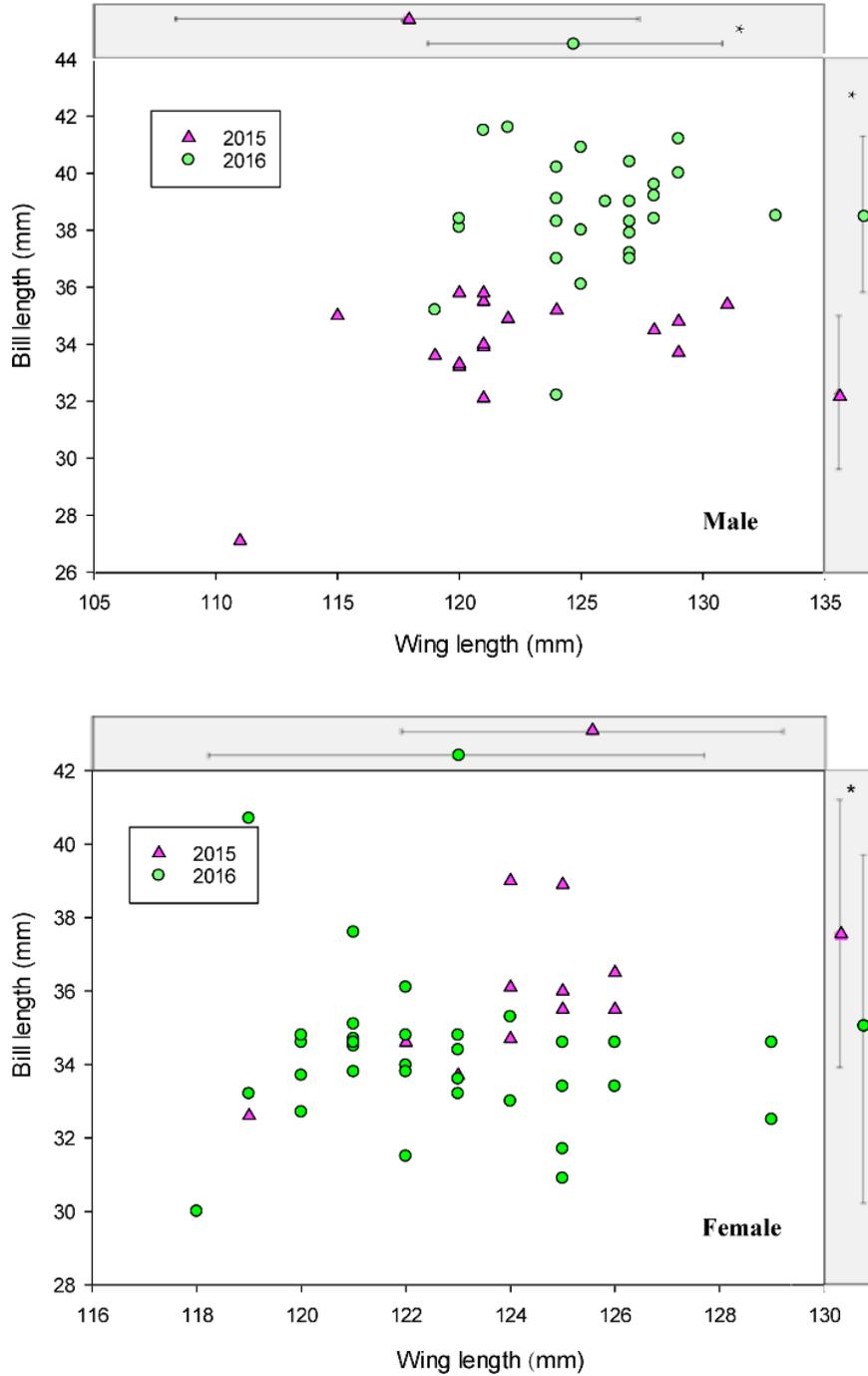
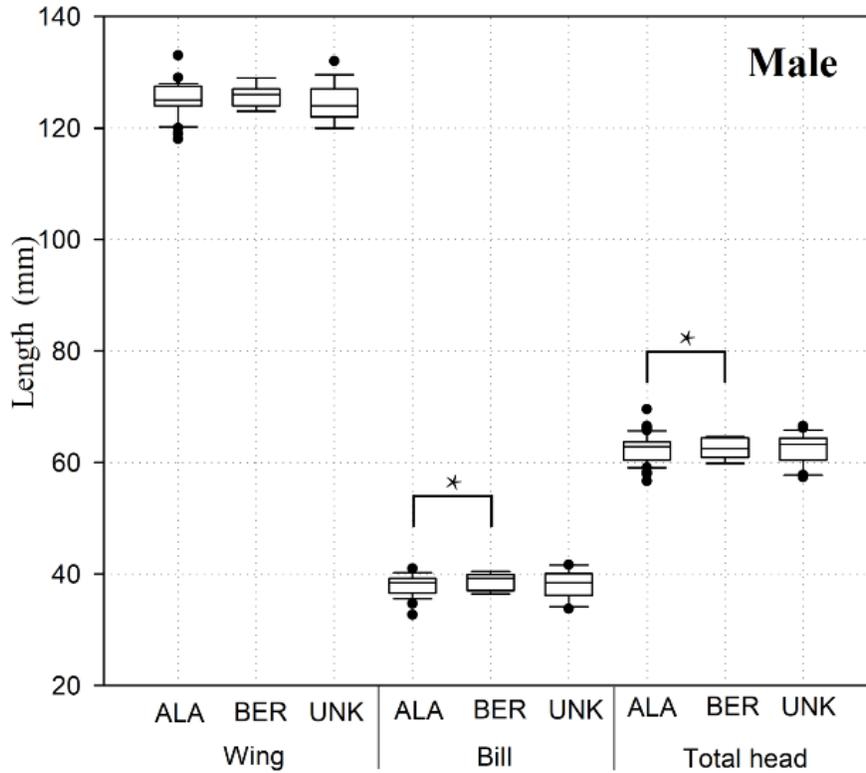


Figure 3 Comparisons of (a) female and (b) male bill and wing lengths of dunlins sampled in Jiangsu in

the autumn migration in 2015 (purple triangles) and 2016 (green circles). The mean \pm standard deviation values for each year are shown at the top and side of each graph. Significance differences between years are indicated by $*P < 0.05$, according to either t -test or Mann–Whitney U-test.

For males, bill and total head lengths were significantly different between Alaskan and Beringian lineages (bill length, $t = 2.172$, $df = 54$, two-tailed $P = 0.034$; total head length, $t = 2.769$, $df = 85$, two-tailed $P = 0.007$) (Fig. 4a). Bill and total head lengths were shorter in the Alaskan lineage than in the Beringian lineage. By contrast, wing length was longer in the Alaskan lineage than in the Beringian lineage. For females, bill and total head lengths were both longer in the Alaskan lineage than in the Beringian lineage.



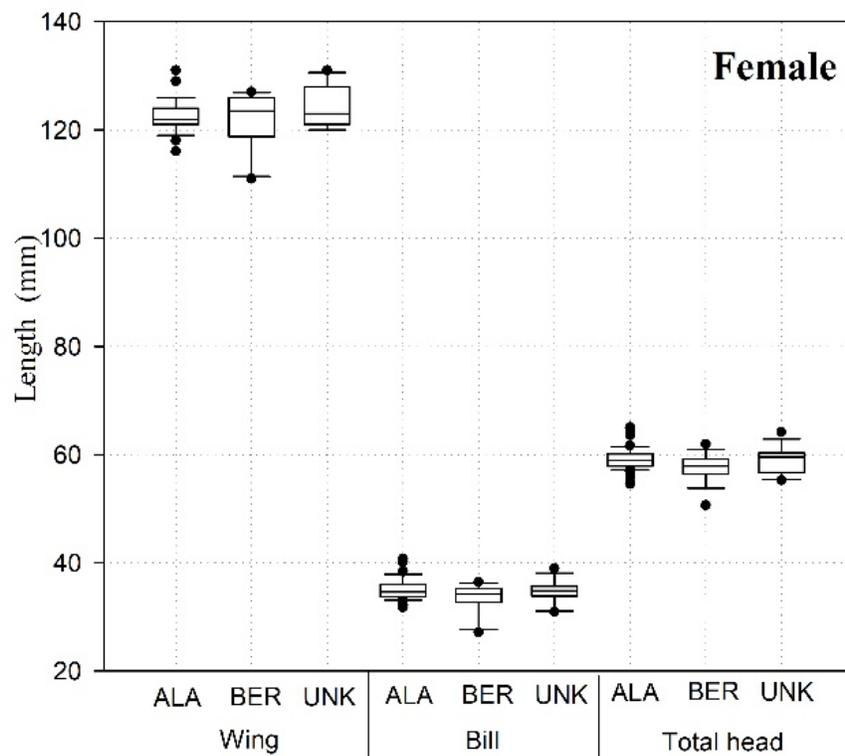


Figure 4 Comparisons of Dunlin (a) male and (b) female wing, bill, and total head lengths from different lineages. ALA, Alaskan lineage; BER, Beringian lineage; UNK, unknown lineages. Significance differences between groups are indicated by * $P < 0.05$, according to either t -test or Mann–Whitney U-test.

Discussion

The study provided new insights into the origins of dunlins migrating along the EAAF and a different perspective on the subspecies composing Dunlin populations on the flyway. In previous studies, how many Dunlin subspecies migrate along the EAAF was not rigorously determined. The combined results of ring recoveries and flag resightings from this study and previous research (Lagassé et al., 2020; Lagassé et al., 2022) indicated that the subspecies *C. a. sakhalina*, *C. a. actites*, *C. a. kistchinski*, and *C. a. arcticola* staged at Jiangsu in autumn. The result of recoveries and resightings suggested that compared with other subspecies, more individuals from *C. a. kistchinski* and *C. a. actites* were staging in southern Jiangsu Province, which is an important site to replenish energy reserves for those individuals from the Kamchatka Peninsula and Sakhalin Island. In addition, recoveries and resightings also provided general indications of the routes of dunlin subspecies along the EAAF (Supporting information Fig. S1).

Identification of *C. a. arcticola* and *C. a. actites* was important, because a recent study showed that survival is relatively low for the *arcticola* subspecies and that the population size of *C. a. actites* is relatively small (Weiser et al. 2018). The population of subspecies *C. a. actites* is thought to number less than 1000 individuals, compared with over 500,000 for the other subspecies (Johnston et al. 2015). Thus, the study indicated that the coastal intertidal flats in the study area are important to the conservation of priority dunlin subspecies.

The application of multiple approaches can improve the effectiveness of population identification and studies of migration in most cases. Because most birds and subspecies of birds have highly similar morphology, it is difficult to accurately determine differences among species or subspecies by one specific method. Resighting and recovery data are one of the many types of data obtained from long-term fieldwork and represent a large

investment. Although molecular methods cannot designate all individuals to one lineage or one subspecies, phylogenetic analysis can substantially increase understanding of dunlin population structure and help to realize approximate migratory phenology. Genetic data and phenotypic characters are both widely used in studies of shorebird phylogeny and taxonomy (Popovic et al. 2019). Phenotypic characters may be useful in probing the composition of populations (Rheindt et al. 2011). Hence, this study combined data of resighting or recovery and morphological and molecular analyses to comprehensively delineate dunlin population structure and migratory connectivity.

The analysis of mtDNA sequence data provided detailed information on the population composition and migratory connectivity of dunlin subspecies at Jiangsu. The haplotypes from Jiangsu had sister relationships with different dunlin subspecies and shared some haplotypes with those subspecies. Haplotypes from Jiangsu suggested the occurrence of six subspecies grouped into two lineages: Alaskan and Beringian. This result indicated that the dunlin population in Jiangsu was composed of different subspecies and that the nonbreeding ranges of those subspecies overlapped substantially in Jiangsu. Similar observations are reported in Portugal (Lopes et al. 2006).

The morphological difference between Alaskan and Beringian lineages is consistent with that among subspecies from the Far East and Alaska. Therefore, individuals in the Alaskan lineage included *C.a. arcticola* and *C. a. pacifica* and individuals in the Beringian lineage included *C. a. actites*, *C. a. kistchinski*, and *C. a. sakhalina*. Similar to the descriptions of dunlin subspecies (Greenwood 1984, 1986), males of subspecies from Alaska had shorter bills and longer wings than those from the Far East, whereas females of subspecies from Alaska had longer bills and wings than those from the Far East.

Although the six subspecies could not be accurately separated, it is plausible that at least four dunlin subspecies previously thought to share winter ranges along the central coast of eastern Asia far-stage in China (*C. a. actites*, *C. a. kistchinski*, *C. a. sakhalina*, and *C. a. arcticola*) (Bentzen et al. 2016, Choi et al. 2010, Miller et al. 2014, Tomkovich 1986). The subspecies *C. a. hudsonia* and *C. a. pacifica* from breeding grounds in North America were unexpected, because they were not thought to stage on the coast of eastern China (Miller et al. 2014). However, in this study, the two subspecies most likely occurred in Jiangsu.

Because of changes in populations, different populations of dunlin might be represented to a different extent in the 2 years. However, distinct clades were not detected between the 2 years, and only 10 haplotypes were shared between the sample periods (Supporting information Fig. S2). This may be due to the indistinct phylogenetic relationships among the six subspecies (Miller et al. 2014). By combining size measurements and molecular genetic analyses, the results of this study highlight the need to reassess the composition and migratory routes of subspecies at the level of subspecies. Morphology can help with an intuitive feeling for the subspecies of dunlin. The morphological data from 2015 and 2016 indicated that bill and wing lengths differed between the 2 years, but those differences were in opposite directions for the two sexes. Dunlin were captured considerably later in autumn 2016 (26 September to 6 October) than in autumn 2015 (12–20 September), so apparent annual differences might be spurious and only represent differences in migration timing among subspecies.

Previous studies demonstrate consistent migration timing among breeding populations, which correlates with geographical locations of breeding grounds (Grattarola et al. 1999, Jahn et al. 2013). In addition, significant differences in morphological characters were observed between Alaskan and Beringian lineages, which can help determine breeding origin. Moreover, compared with the number of Beringian and Canadian individuals, the number of Alaskan individuals was greater in 2016 compared to 2015. This difference might be a possible reason for the difference in characters between the two years. However, the differences between the two lineages could not entirely explain the differences in individuals between the sample periods. This result might be explained by variable proportions of different lineages or a difference in the timing of migration of subspecies.

The above findings provided additional evidence on the importance of the Jiangsu mudflats in providing migratory connectivity for subspecies and strengthened the case that protective measures are needed. There

is some evidence that populations of some priority dunlin subspecies that stage at Jiangsu, such as *C. a. arctica*, *C. a. actites*, and *C. a. sakhalina*, are declining (Johnston et al. 2015, Weiser et al. 2018). The conservation of stopover sites to maintain the connectivity of migration is important for long-term population persistence of subspecies from the Beringian realm (Soikkeli 1967). Conversion of intertidal shorebird habitats to land for industry and farming, construction of China's new great wall along the seacoast, and invasion of mudflats by exotic cordgrass (*Spartina* sp.) all contribute to rapid losses in the extent of intertidal shorebird habitats (Li et al. 2022, Ma et al. 2014). Although measures to protect the Jiangsu mudflats and their enormous contribution to the future of shorebird populations should be implemented, additional conservation action is still required. This study draws attention to the huge geographical area from which migrating dunlin subspecies using the Jiangsu coast are drawn. Further research of this type is needed that focuses on numbers of individuals and population trends of different dunlin subspecies staging in Jiangsu and the timing of their migrations. The approach used in this study should also be extended to other shorebird species using this important stopover site.

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Conflicts of interest No conflicts of interest or competing interests.

Permits Dunlin capture, tagging, and sampling were conducted under a special methods permit from the Jiangsu Forestry Bureau and National Bird Ring Center.

Author contributions

Wei Liu : Conceptualization (coequal); Data curation (lead); Formal analysis (lead); Writing – original draft (lead). **David S. Melville** : Supervision (supporting); Writing – Review and editing (coequal). **Qing Chang** : Conceptualization (coequal); Supervision (supporting); Writing – Review and editing (coequal).

Data availability statement

Data are freely available from Dryad: <https://datadryad.org/stash/share/J2j3OLE-YmbIFgD6cTNYm8LkyiY06HWwFE3F7fnoPE>.

Supporting information

The supporting information associated with this article is available with the online version.

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