

## Mitochondrial DNA suggests independent evolutionary history and population decline of the Menzbir's pipit (*Anthus [gustavi] menzbieri*)

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**Abstract** In this study we use mtDNA ND2 gene (1041 bp) to evaluate the relationship between Menzbir's (*Anthus [gustavi] menzbieri*) and Pechora (*A. [g.] gustavi*) pipits. Menzbir's pipit is listed in the regional Red Data Book as a distinct, rare species with a small range. We obtained 18 Pechora pipit samples from two localities and 8 Menzbir's pipit samples from a single locality. Sequences of the two taxa appear reciprocally monophyletic and are separated by 6 substitutions (0.6% divergence). Differences between the taxa explained 62.4% of the variation in our dataset. Differences among individuals within localities explained 34.8%, whereas differences between the two Pechora pipit localities explained only 2.8%. Mismatch distributions suggest that unlike the Pechora pipit localities, which either have experienced recent population growth or sustain a stable population size, the Menzbir's pipit population may be declining. Our results suggest distinct taxonomic and conservation status for the Menzbir's pipit.

**Keywords** Mitochondrial DNA · ND2 · Pechora pipit · *Anthus gustavi* · Menzbir's pipit · Mismatch distribution

### Introduction

Menzbir's pipit (*Anthus [gustavi] menzbieri*) is a poorly known passerine breeding in the Amur River valley

between 130–135°E and lowlands around Lake Khanka in the Russian Far East (Fig. 1). Its taxonomic status is uncertain with most authors treating it as a subspecies of the Pechora pipit (*A. gustavi*) whose range extends across high latitudes from north eastern Europe to the Bering Sea (Dickinson 2003; Stepanyan 2003). Phenotypic differences between the two taxa are limited to the size of light edges on the contour feathers of the back. Pechora pipits have larger edges and appear lighter than Menzbir's pipits (Stepanyan 2003).

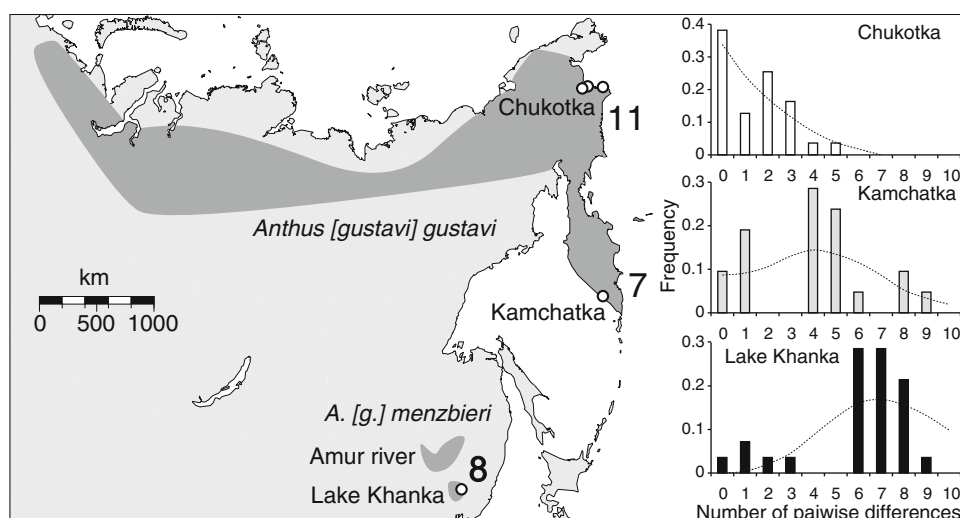
Despite the limited phenotypic differences, Dickinson (2003) and Stepanyan (2003) question the conspecific status of Pechora and Menzbir's pipits. Stepanyan (2003) did not cite any reason for his uncertainty, whereas Dickinson (2003) cited Leonovich et al. (1997). The latter provided no data to support their argument. Rather, they based it on the original description of the Menzbir's pipit (Shul'pin 1927) which states that the new pipit, despite being closely related to the Pechora pipit, differs greatly and unless intermediate forms are found, it can be considered a distinct species. Leonovich et al. (1997) point to the lack of such intermediates and show the sonograms of a single song and call of the Menzbir's pipit and three songs and one call of the Pechora pipit. Although these recordings appear different, there is no analysis of these differences or the variation within taxa. Nonetheless, the regional Red Data Book of Primorskiy Krai (Litvinenko et al. 2002) lists Menzbir's pipit as a distinct species and assigns it category 3 status—"rare" (taxa and populations that have low numbers and small ranges or are sporadically distributed across large territories).

Little data are available to support such a taxonomic treatment or the listing of Menzbir's pipit as "rare" rather than assigning it another conservation category. This lack of data resulted in omission of the Menzbir's pipit from the

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**Fig. 1** Ranges extrapolated from (Stepanyan 2003), sample sizes, localities, and mismatch distributions of Pechora and Menzbir's pipits. Dotted lines represent distributions expected under the sudden population expansion model



IUCN (<http://www.iucnredlist.org/>) and BirdLife International (<http://www.birdlife.org/datazone/species/index.html>) lists, and from the Russian federal Red Data Book (<http://www.biodat.ru/db/rb/rb.php?src=0&grp=7>). When included into Pechora pipit, Menzbir's pipit becomes part of a species with a large range whose global population appears stable.

We use mitochondrial ND2 gene sequences to determine the level of differentiation between the Pechora and Menzbir's pipits and to explore their recent demographic history. We hope that our results will generate interest in the ecology and conservation of the Menzbir's pipit whose habitat experiences strong pressure from human agricultural activities across its range (Leonovich et al. 1997).

## Materials and methods

We obtained 18 tissue samples of breeding Pechora pipits from Chukotka ( $n = 11$ ) and Kamchatka (7), and 8 samples of Menzbir's pipits from the Lake Khanka area (Fig. 1) from museum collections (Table 1). The distances between Kamchatka and the other two localities were similar, 1764 km to Chukotka and 1922 km to Khanka. However, the two former localities lie within the range of the Pechora pipit, but the distance between Kamchatka and Khanka represents the gap between the ranges of the two taxa.

Total genomic DNA extraction, PCR profile and primers for amplification and sequencing of the mitochondrial ND2 gene (1041 bp) followed Drovetski et al. (2004). The PCR fragments were sequenced directly on an ABI-3730 sequencer using Big Dye chemistry (Applied Biosystems, Foster City, CA, USA).

Sequences were aligned in Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Unique haplotypes

were identified using DNAsp 5 (Librado and Rozas 2009). To compare levels of genetic variation among localities we calculated haplotype ( $H_d$ ) and nucleotide ( $\pi_n$ ) diversity and their standard deviations (SD) using Arlequin 3.11 (Excoffier et al. 2005). To test the neutrality of ND2 evolution or demographic expansion we conducted Tajima's  $D$  (Tajima 1989, 1996) test for each locality using Arlequin and R2 (Ramos-Onsins and Rozas 2002) and McDonald–Kreitman ( $M-K$ ; McDonald and Kreitman 1991) tests using DnaSP. To determine levels of genetic differentiation among localities we computed pairwise  $F_{st}$  values and to determine the degree of the divergence between taxa we conducted AMOVA (Excoffier et al. 1992) using Arlequin. The hierarchical structure in our AMOVA was composed of three levels: subspecies (Pechora and Menzbir's pipits), localities within subspecies (Pechora pipit: Kamchatka and Chukotka, Menzbir's pipit: Khanka), and individuals within localities. For both  $F_{st}$  calculations and AMOVA we used uncorrected differences among haplotypes. The haplotype network was constructed using TCS (Clement et al. 2000). To compare the demographic history of populations we plotted the mismatch distribution for each sampling locality. We used Arlequin to calculate the Harpending's raggedness index (HRI; Harpending 1994) and the sum of squared deviations (SSD; Excoffier et al. 2005) for each mismatch distribution to test the fit of the sudden population expansion model.

## Results

DNAsp identified 16 unique haplotypes among 26 birds. The TCS network identified two geographically and taxonomically concordant clades separated by 6 substitutions (0.6% divergence). One clade combined all Menzbir's pipits whereas the other combined all Pechora pipits (Fig. 2). Only two of eight Menzbir's pipits shared the same haplotype.

**Table 1** Sample IDs, institutions housing voucher specimens, localities, dates, sex, and NCBI accession numbers for birds used in this study

Tissue ID	Museum	Lat., °N	Long., °E	Date	Sex	NCBI
Chukotka						
B1	MSUZM	63.07	179.34	Breeding	?	HM538381
csw4477	UWBM 43937	64.12	178.25	11-Jul-1992	M	HM538371
csw4485	UWBM 43945	64.12	178.25	11-Jul-1992	F	HM538372
csw4491	UWBM 43951	64.12	178.25	11-Jul-1992	F	HM538373
csw4498	UWBM 43958	64.12	178.25	11-Jul-1992	M	HM538374
evn441	BMUM	64.42	177.40	14-Jun-2003	M	HM538375
svd2855	UWBM 82240	64.42	177.40	12-Jun-2003	F	HM538376
svd2875	BMUM	64.42	177.40	13-Jun-2003	M	HM538377
svd2876	BMUM	64.42	177.40	13-Jun-2003	M	HM538378
svd2877	BMUM	64.42	177.40	13-Jun-2003	M	HM538379
svd2904	UWBM 82251	64.42	177.40	14-Jun-2003	M	HM538380
Kamchatka						
evn892	SDM	52.81	156.43	4-Jun-2009	M	HM538382
evn909	SDM	52.81	156.43	6-Jun-2009	M	HM538383
evn910	SDM	52.81	156.43	6-Jun-2009	M	HM538384
ivf910	SDM	52.81	156.43	3-Jun-2009	M	HM538385
svd4405	SDM	52.81	156.43	3-Jun-2009	F	HM538386
svd4406	SDM	52.81	156.43	3-Jun-2009	M	HM538387
svd4407	SDM	52.81	156.43	3-Jun-2009	M	HM538388
Lake Khanka						
rya790	UWBM 75485	44.94	132.92	15-Jun-2002	F	HM538389
rya791	UWBM 75486	44.94	132.92	15-Jun-2002	M	HM538390
rya820	UWBM 75515	44.94	132.92	16-Jun-2002	M	HM538391
rya821	UWBM 75516	44.94	132.92	16-Jun-2002	F	HM538392
rya827	UWBM 75522	44.94	132.92	16-Jun-2002	?	HM538393
rya828	UWBM 75523	44.94	132.92	16-Jun-2002	F	HM538394
rya829	UWBM 75524	44.94	132.92	16-Jun-2002	F	HM538395
rya861	UWBM 75556	44.94	132.92	17-Jun-2002	F	HM538396

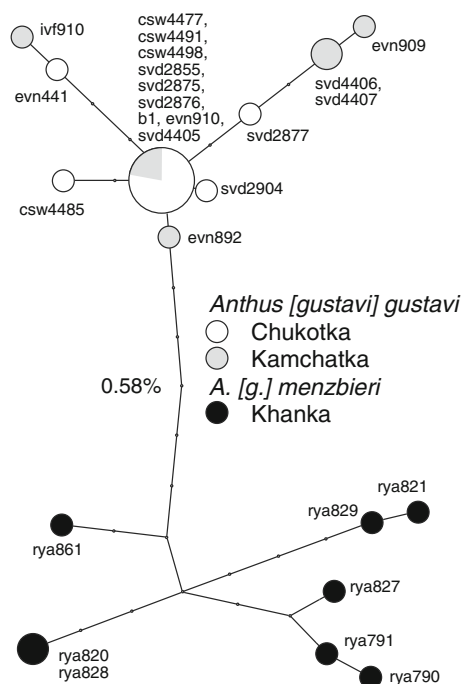
*UWBM* University of Washington Burke Museum of Natural History and Culture (Seattle, USA), *BMUM* University of Minnesota Bell Museum of Natural History (St. Paul, USA), *SDM* State Darwin Museum (Moscow, Russia), *MSUZM* Moscow State University Zoological Museum (Moscow, Russia)

The most common Pechora pipit haplotype was shared by 7 of 11 Chukotka birds and two of seven Kamchatka birds.

Pairwise  $F_{st}$  values and AMOVA results provided additional support for the strong differentiation between taxa.  $F_{st}$  values between Khanka and either of the northern localities were large (Chukotka/Khanka  $F_{st} = 0.656$ , Kamchatka/Khanka  $F_{st} = 0.569$ ) and significant ( $P < 0.0001$  for both), whereas the  $F_{st}$  value for Chukotka and Kamchatka was 4 times smaller and only marginally significant ( $F_{st} = 0.145$ ,  $P = 0.045 \pm 0.020$ ). Our AMOVA indicated that differences between Menzbir's and Pechora pipits accounted for 62.4% ( $P < 0.0001$  for polymorphic loci only) of the variation in our dataset. Differences among individuals within localities accounted for 34.8% ( $P < 0.0001$ ) of the variation, whereas differences between Chukotka and Kamchatka accounted only for 2.8% ( $P = 0.005$ ).

The M–K test did not find a significant difference between ratios of replacement to synonymous substitutions among fixed differences between (0:6) and among polymorphic sites within (5:21) taxa (two-tailed Fisher's exact  $P = 0.555$ ). This suggests that the mtDNA divergence between Menzbir's and Pechora pipits was not significantly affected by selection.

Genetic diversity varied widely among localities. Birds from Khanka had the largest values for both  $Hd$  ( $0.964 \pm 0.072$ ) and  $\pi_n$  ( $0.005764 \pm 0.003501$ ). Birds from Kamchatka had high  $Hd$  ( $0.905 \pm 0.103$ ) and moderate  $\pi_n$  ( $0.003842 \pm 0.002500$ ). Birds from Chukotka had much lower  $Hd$  ( $0.618 \pm 0.164$ ) and  $\pi_n$  ( $0.001397 \pm 0.001034$ ) than other localities. These data suggest that the Khanka population is dominated by divergent haplotypes of low frequency, whereas Chukotka is dominated by one common



**Fig. 2** TCS network of Pechora and Menzbir's pipit ND2 haplotypes

haplotype and other haplotypes are closely related to it. Kamchatka birds carry many low frequency haplotypes of different divergence levels. These patterns of mtDNA diversity are well represented by the mismatch distributions (Fig. 1). The distribution for Chukotka had the lowest mean mismatch (1.455) and a single peak at 0 differences. The distribution for Kamchatka was ragged with a mean of 4, tallest peak at 4, and two lower peaks at 1 and 8. The distribution for Khanka was left-skewed with major peaks at 6 and 7 and mean of 6. The SSD (0.025, 0.083, and 0.077, respectively) and HRI (0.107, 0.181, and 0.124, respectively) were not significant for any locality, failing to reject the sudden population expansion model. Tajima's  $D$  value ( $-1.934$ ) was significant ( $P = 0.006$ ) and  $R_2$  value (0.132) was marginally significant ( $P = 0.057$ ) only for Chukotka, further supporting population expansion in that northernmost locality. Tajima's  $D$  and  $R_2$  values for Kamchatka and Khanka were not significant. These mismatch distributions and test results indicate that only Chukotka appears to have experienced significant population growth in the recent past.

## Discussion

Our haplotype network, AMOVA results, and pairwise  $F_{st}$  values suggest significant mtDNA differentiation between Menzbir's and Pechora pipits. Despite the similarity of the geographic distance between Kamchatka and the other two localities, Kamchatka birds were placed in the same clade

with Chukotka samples, but Khanka birds formed a distinct clade. The differences between these clades accounted for nearly two-thirds of the variation in our data, with the other third accounted for by differences among individuals within localities. The pairwise  $F_{st}$  values between Khanka and the other two localities were four times greater than the  $F_{st}$  value between Kamchatka and Chukotka.

The 0.6% divergence between Menzbir's and Pechora pipits indicates that these taxa have been isolated since the upper Middle Pleistocene if the most conservative evolutionary rate of 2% divergence per million years is employed, or since the Late Pleistocene if faster rate estimates are assumed (Lovette 2004). Therefore, it is likely that climatic oscillations of the Pleistocene resulted in the divergence of these taxa as was first suggested by Leonovich et al. (1997).

The divergence between Menzbir's and Pechora pipits closely resembles the peripatric speciation in the Holarctic avian subfamily Tetraoninae in which isolation of small, populations south of the large continuous range appears to be the most common geographic mode of speciation (Drovetski 2003). The differentiation between Menzbir's and Pechora pipits is expected to increase in the future because the continuing postpleistocene climate warming is likely to increase the gap between the tundra habitat of the Pechora pipit and relictual wetland habitat of the Menzbir's pipit. Regardless of the Menzbir's pipit taxonomic treatment, its genetic distinctiveness, small range, and the agricultural conversion of its habitat should raise conservation concerns for this poorly studied taxon. Leonovich et al. (1997) noted the especially devastating effect of the 1986 drainage of the wetlands surrounding Lake Khanka on the population density and distribution of the Menzbir's pipit which became restricted to a single small area in the region.

The comparison of mismatch distributions reinforces this conservation concern. The northernmost of the three localities examined (Chukotka) had a unimodal distribution with a peak at 0 differences. This is consistent with the signature of recent population expansion (Rogers and Harpending 1992). The significant and negative Tajima's  $D$  value, marginally significant  $R_2$  value, and non-significant SSD and HRI values further support the inference of recent population growth in Chukotka. Although we were unable to reject neither the sudden population expansion model nor stable population size for Kamchatka or Khanka, the ragged mismatch distribution of the Kamchatka sample suggests that this southern Pechora pipit population may be at equilibrium (Rogers and Harpending 1992). In contrast, the left-skewed mismatch distribution for the Khanka population may suggest a population decline. The theoretical expectation for a mismatch distribution in a population experiencing a bottleneck is raggedness with peaks

at large values (Rogers and Harpending 1992). Studies of the Guadalupe fur seal (*Arctocephalus townsendi*; Weber et al. 2004) and greater prairie-chicken (*Tympanuchus cupido*; Johnson et al. 2007) confirmed these expectations empirically. Prior to known bottlenecks, populations had unimodal or ragged distributions with central peaks and  $HRI = 0.009$  and  $0.013\text{--}0.688$ , respectively. After the bottlenecks, the mismatch distributions became left-skewed with greater HRIs ( $0.101$  and  $0.045\text{--}0.720$ , respectively), similar to that of the Menzbir's pipit ( $HRI = 0.124$ ). Both empirical studies also found that Fu's  $F_s$  and Tajima's  $D$  values for the bottlenecked populations were not significantly different from 0, similar to the Menzbir's pipit.

In conclusion, our study argues for a detailed evaluation of the conservation and taxonomic status of the Menzbir's pipit. Although it is currently classified as category 3 by the regional Red Data Book (Litvinenko et al. 2002), our results suggest that Menzbir's pipit should be upgraded to category 2—"declining" (taxa and populations with declining numbers which could become endangered if the factors causing their decline continue to affect them) and listed in the Russian federal Red Data Book.

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