View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Munin - Open Research Archive

OPEN ACCESS Freely available online

PLOS ONE

Limited Phylogeographic Signal in Sex-Linked and Autosomal Loci Despite Geographically, Ecologically, and Phenotypically Concordant Structure of mtDNA Variation in the Holarctic Avian Genus *Eremophila*

Sergei V. Drovetski[1]*, Marko Raković[2], Georgy Semenov[3], Igor V. Fadeev[4], Yaroslav A. Red'kin[5]

1 Department of Natural History, Tromsø University Museum, University of Tromsø – The Arctic University of Norway, Tromsø, Norway, **2** Department of Vertebrate Zoology, Natural History Museum Belgrade, Belgrade, Serbia, **3** Thematic Group on Bird Ecology, Institute of Systematics and Ecology of Animals of Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia, **4** Department of Collections, State Darwin Museum, Moscow, Russia, **5** Department of Ornithology, Zoological Museum of Moscow State University, Moscow, Russia

Abstract

Phylogeographic studies of Holarctic birds are challenging because they involve vast geographic scale, complex glacial history, extensive phenotypic variation, and heterogeneous taxonomic treatment across countries, all of which require large sample sizes. Knowledge about the quality of phylogeographic information provided by different loci is crucial for study design. We use sequences of one mtDNA gene, one sex-linked intron, and one autosomal intron to elucidate large scale phylogeographic patterns in the Holarctic lark genus *Eremophila*. The mtDNA ND2 gene identified six geographically, ecologically, and phenotypically concordant clades in the Palearctic that diverged in the Early - Middle Pleistocene and suggested paraphyly of the horned lark (*E. alpestris*) with respect to the Temminck's lark (*E. bilopha*). In the Nearctic, ND2 identified five subclades which diverged in the Late Pleistocene. They overlapped geographically and were not concordant phenotypically or ecologically. Nuclear alleles provided little information on geographic structuring of genetic variation in horned larks beyond supporting the monophyly of *Eremophila* and paraphyly of the horned lark. Multilocus species trees based on two nuclear or all three loci provided poor support for haplogroups identified by mtDNA. The node ages calculated using mtDNA were consistent with the available paleontological data, whereas individual nuclear loci and multilocus species trees appeared to underestimate node ages. We argue that mtDNA is capable of discovering independent evolutionary units within avian taxa and can provide a reasonable phylogeographic hypothesis when geographic scale, geologic history, and phenotypic variation in the study system are too complex for proposing reasonable *a priori* hypotheses required for multilocus methods. Finally, we suggest splitting the currently recognized horned lark into five Palearctic and one Nearctic species.

Citation: Drovetski SV, Raković M, Semenov G, Fadeev IV, Red'kin YA (2014) Limited Phylogeographic Signal in Sex-Linked and Autosomal Loci Despite Geographically, Ecologically, and Phenotypically Concordant Structure of mtDNA Variation in the Holarctic Avian Genus *Eremophila*. PLoS ONE 9(1): e87570. doi:10.1371/journal.pone.0087570

Editor: Dan Mishmar, Ben-Gurion University of the Negev, Israel

Received October 16, 2013; **Accepted** December 23, 2013; **Published** January 30, 2014

Copyright: © 2014 Drovetski et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by Fundação para a Ciência e a Tecnologia (http://www.fct.pt/) grant PTDC/BIA-BEC/103435/2008 and Fundação Calouste Gulbenkian (http://www.gulbenkian.pt/Institucional/en/Homepage), Portugal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sdrovetski@gmail.com

Introduction

Avise et al. [1] introduced the term "phylogeography" to describe the geographic structuring of mtDNA lineages. A little over a decade later, Avise [2] defined phylogeography as a discipline focused on "the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species". This discipline is rooted in empirical studies of geographic variation of mtDNA and many phylogeographic studies still use mtDNA markers.

The reasons for the extensive use of mtDNA in phylogeography are well-known: fast substitution rate, lack of recombination, small effective population size resulting in fast lineage sorting and high sensitivity to demographic events. These characteristics, combined with the technical ease of collecting large amounts of sequence data that do not require phasing, make mtDNA the most popular marker for phylogeographic inquiry [2–5].

MtDNA has been utilized in a large number of avian phylogeographic studies routinely identifying various degrees of intraspecific lineage sorting or non-random geographic variation of genetic diversity such as geographically concordant clades, isolation-by-distance, geographic clines of genetic diversity, etc. [2,4].

MtDNA, however, represents only the matrilineal history of avian taxa. Yet, with a few exceptions, females are the dispersing sex in birds - a higher proportion of females disperse from the natal area than males and females disperse over longer distances than males [6]. Thus, maternally inherited mtDNA should be a reliable indicator of phylogeographic patterns among sampled localities.

MtDNA may be susceptible to lineage sorting, branch length stochasticity, and to introgression across taxonomic and biogeographic borders [7–10]. The presence of interspecific mtDNA far away from the current hybrid zones has been identified in some avian species [11], but sampling of closely related taxa readily identifies such cases. Furthermore, increasing the sample size and number of characters can also reduce the lineage sorting and branch length stochasticity. This reduction results from sampling of many different haplotypes that increases tree stemminess (the ratio of internal branch lengths to terminal branch lengths) and helps to differentiate the distance between clades from the intraclade variation. Greater stemminess also increases tree resolution and thus, decreases the topological stochastisity [12].

The use of nucleotide sequences from multiple independent nuclear loci is advocated as a better alternative to the use of mtDNA in avian phylogeography [10,13]. The multi-locus approach widely samples the genome and is predicted to eliminate the lineage sorting and branch length stochasticity, and to increase the resolution of phylogeographic reconstructions [10]. It is also predicted to eliminate the need for sampling of many individuals as long as many loci are sampled [14].

Little empirical evidence, however, supports the utility of multilocus sequence data for avian phylogeography [5]. For example, in a multi-locus study of the red-backed fairy wren (*Malurus melanocephalus*), 35 nuclear loci (almost 15,000 bp/individual) failed to recover phylogeographic structure which was readily recovered with only 467 bp of mtDNA [15]. The authors had to use the phylogeographic structure recovered with their mtDNA data to group individuals for estimating population parameters and levels of gene flow with their nuclear sequences. The isolation with migration analysis [16] based on the mtDNA-guided division produced equal time since divergence between neighboring regions, and the authors again had to resort to mtDNA data to gain "additional perspective on the divergence times" [15].

The red-backed fairy wren study used 30 individuals despite that it focused on taxa with limited geographic variation distributed across a small portion of the smallest continent and the authors emphasized sampling loci over individuals [15]. However, many avian species inhabiting northern continents have very large ranges and population sizes, and exhibit considerable geographic variation. Phylogeographic studies of such taxa demand large sample sizes for sufficient coverage of their ranges and phenotypic variation. Performance of different loci in such challenging conditions becomes important for efficient study design.

In this study we compare the performance of three independent loci (one each of mitochondrial, Z-chromosome linked, and autosomal) individually and of their combined analysis to elucidate the large scale geographic pattern of genetic variation in a small Holarctic genus *Eremophila* (Passeriformes: Alaudidae).

The genus *Eremophila* (Aves: Alaudidae) includes only two currently recognized species - the Temminck's lark (*E. bilopha*) and horned lark (*E. alpestris*). The former species is monotypic and inhabits a narrow belt of rocky deserts following the coastal outline of north Africa and the Middle East from westernmost Mauritania in the west to central Iraq in the east [17]. In contrast, the horned lark is a Holarctic and highly polytypic species with over 40 subspecies [18,19]. It is the only lark species that has a large Holarctic breeding range whereas the rest of the family is restricted to the Old World. The habitats of the horned lark include arctic and alpine tundra, arid lands with sparse vegetation, and agricultural fields [17,20].

Nearly two thirds of horned lark subspecies are described from the New World where birds vary primarily in size, back color, and intensity of yellow color on the head, throat, and upper chest [20]. In the Old World, in addition to size and color variation, some alpine subspecies in the central Palearctic are distinguished by a much larger black breast patch which connects with black cheek patches [17]. The remarkable phenotypic variation and large Holarctic breeding range resulted in the original description of many horned lark subspecies, especially those inhabiting the Old World, as distinct species [21].

The taxonomic treatment of the horned lark resembles the taxonomy of the former winter wren (*Troglodytes troglodytes*), which until recently had been treated as a single Holarctic species with over 40 subspecies [18,19]. However, the survey of the winter wren mtDNA identified a number of deeply divergent, geographically concordant clades in each northern continent [22]. Some of these clades are now recognized as distinct species - the Pacific wren (*Troglodytes pacificus*) in the Pacific Northwest of North America, Winter Wren (*T. hiemalis*) in eastern and northern North America, and the Eurasian wren (*T. troglodytes*) in the Palearctic [23]. Many other Holarctic avian species appear to have divergent Nearctic and Palearctic clades, especially those inhabiting forest habitats [24].

Although the horned lark is not a forest species, recent studies indicate that it may contain deep intra- and intercontinental phylogeographic structuring which remains unrecognized by current taxonomy. A study of the status of the streaked horned lark (*E. a. strigata*) identified three divergent, geographically concordant mtDNA clades just in western North America [25]. Each of these clades included multiple subspecies. These clades formed a monophyletic group with respect to the two Palearctic horned larks used as outgroups, suggesting the possibility of divergence between Nearctic and Palearctic birds. The intercontinental differentiation of horned larks was also supported by a study of divergence between Scandinavian and Nearctic populations of birds based on a fragment of mtDNA CO1 gene [26]. A recent study of the phylogeny of the family Alaudidae found deep divergence among horned lark mtDNA Cytochrome-*b* (cyt-*b*) haplotypes sampled in distant parts of the species range [27]. Furthermore, these divergent haplotypes were paraphyletic with respect to the single cyt-*b* haplotype of the Temminck's lark and Palearctic haplotypes were paraphyletic to Nearctic haplotypes. Therefore, available molecular data argues for the need of a re-evaluation of the species limits within *Eremophila*.

Materials and Methods

Ethics Statement

This study did not require ethical approval in our institutions because we used samples loaned to us by public museums or universities (Appendix S1) who comply with relevant regulations for acquisition and curation of their collections.

We used a total of 286 horned lark samples, 5 samples of Temminck's lark, and 3 samples of the greater short-toed lark *(Calandrella brachydactyla;* designated as outgroups) obtained from museum collections or academic institutions (Fig. 1; Appendix S1). In our initial Neighbor-Joining analysis of mtDNA ND2 sequences, we tested the following lark genera as potential outgroups: *Alauda, Gallerida, Lullula*, and *Melanocorypha*, but *Callandrella* appeared to be the most closely related to *Eremophila*. This was also confirmed by a recent study of the Alaudidae phylogeny [27].

Most of the samples consisted of muscle tissue preserved in 96% ethanol and have associated voucher specimens in museum collections (Appendix S1). However, the samples from British

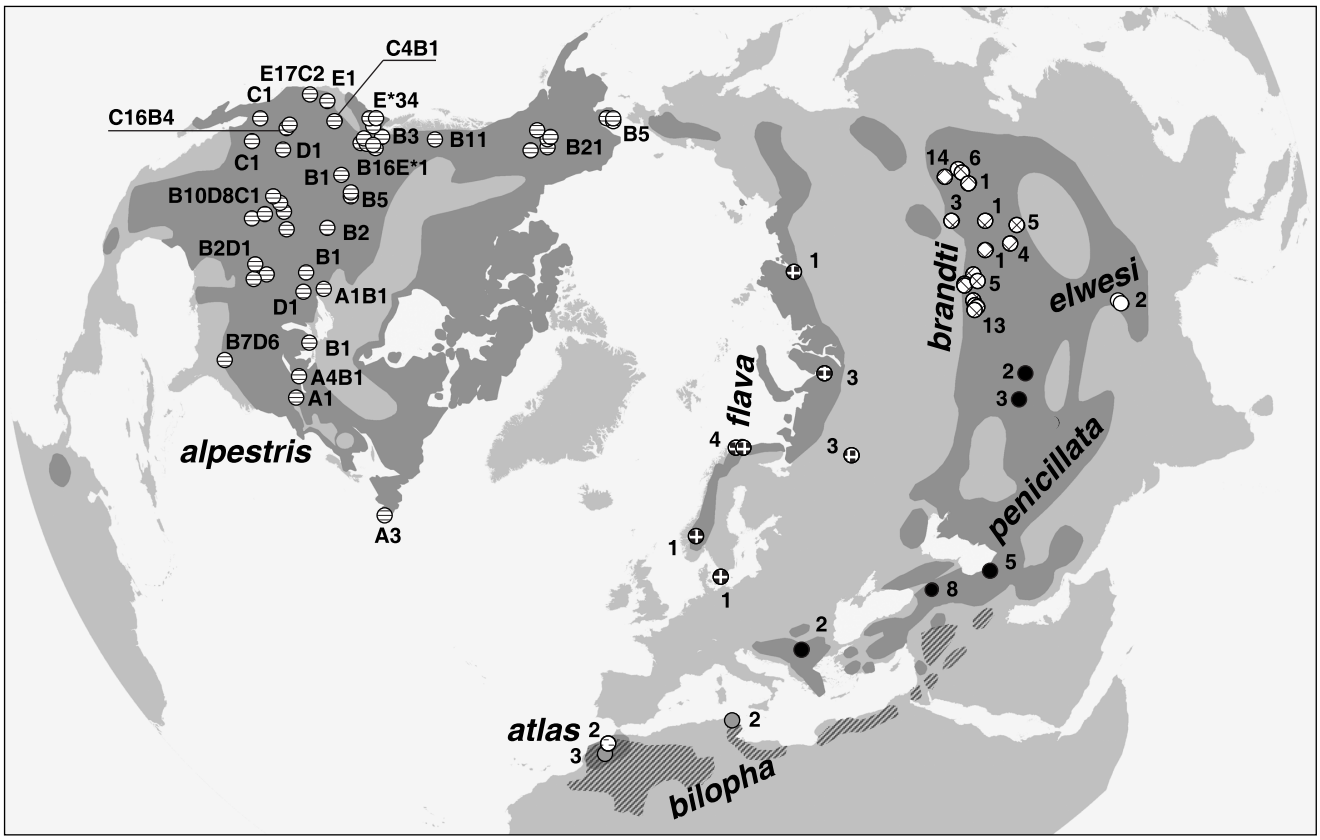

Figure 1. Sampling localities of *Eremophila* larks. MtDNA clades sampled in each locality are identified by clade names from Fig. 2. The numbers following Nearctic clade names indicate their sample sizes. Dark gray areas show ranges of the currently recognized horned lark (solid) and Temminck's lark (striped). The following sources for lark distribution data were used to create this map: [20,21,53,54].
doi:10.1371/journal.pone.0087570.g001

Columbia, Canada were growing contour feathers and samples from Georgia, USA were amnion harvested from recently hatched eggs (1 per nest). Both the feathers and amnion tissue were preserved in 96% ethanol.

Genomic DNA was extracted using the JETQUICK Tissue DNA Spin Kit (Genomed, Loöhne, Germany) or DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols. We obtained complete mtDNA ND2 gene sequences for 294 of 296 larks sampled for this study. We used 100 ND2 sequences available in GeneBank (accession numbers DQ187388– DQ187487) [25] and two recently published sequences of the streaked horned lark (*E. a. strigata*) [28]. We sequenced 194 additional samples (GeneBank accession numbers: KF735311 - KF735504) using primers and protocols described by Drovetski et al. [29].

We also sequenced intron 9 of the Z chromosome specific Aconitase 1 gene (ACO1I9, 983 bp; GenBank accession numbers: KF735211 - KF735310) for a subset of 59 individuals and autosomal intron 1 of the rhodopsin gene (RHOI1, 922 bp; GenBank accession numbers: KF735505 - KF735628) for a subset of 62 birds. Both subsets included multiple representatives of all ND2 clades. ACO1 was amplified using primers ACO1-I9F2 (CTCCTCTCAGGATCCAGACTT) and ACO1-I9R2 (CAACTTTGTCCTGGGGTCTTT) and annealing temperature 55°C [30]. RHOI1 was amplified using primers RHO-I1F (TGCTACATCGAGGGCTTCTT) and RHO-I1R (CGAGTGACCAGAGAGCGATT) and annealing temperature 56°C [31]. PCR fragments were sequenced in both directions on an ABI 3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). The sequences were aligned automatically in Sequencher 5.0.1 (Gene Codes Corporation, Ann Arbor, MI) and verified manually to ensure consistent alignment of indels.

In heterogametic individuals whose alleles differed in length, the alleles were identified by subtracting the complimentary sequence of the allele without the indel from the double peaks in their chromatogram [32]. Alleles of heterogametic individuals that had the same length but contained multiple nucleotide differences we resolved using PHASE 2.1.1 [33]. We conducted two independent PHASE runs. The first 500 interactions were discarded as burn-in. The following 5000 iterations used a thinning interval of 10.

We used *BEAST 2.0.2 [34] to reconstruct multi-locus species and locus-specific trees and to estimate divergence times among lineages. We used the mean rate of sequence evolution and associated 95% confidence interval (CI) reported by [35] for ND2 (2.9×10^{-2} substitutions/site/Ma [$2.4 - 3.3\times10^{-2}$]). For ACO1I9 and RHOI1 we allowed rates to be estimated relative to that of ND2. These estimate were 9.8×10^{-3} substitutions/site/Ma (95% CI: $0.6 - 1.4\times10^{-2}$) and 5.0×10^{-3} substitutions/site/Ma (95% CI: $3.1 - 6.9\times10^{-3}$) respectively.

We used the Bayesian information criterion (BIC) implemented in jModelTest (Posada 2008) to select substitution models for the *BEAST analyses. For ND2 jModelTest selected TrN+G submodel of the generalized time reversible (GTR) model [36] where transversions are weighted equally with discrete-gamma (G) model of substitution rates across sites [37]. For ACO1 jModelTest selected 010220 + I submodel of GTR model with the proportion

of invariable sites (I) included. For RHOI1 jModelTest selected K80 [38] with the proportion of invariable sites (I) included (K80 + I). We incorporated a Yule process speciation prior for our *BEAST analysis. To select the appropriate molecular clock prior, we conducted two independent runs for each locus. In one run we used a strict clock prior and in the other relaxed lognormal clock prior. We then conducted a maximum likelihood ratio test [39] to determine whether the strict clock tree likelihood was significantly worse than the relaxed clock tree likelihood. Because MLRT was not significant (all P values > 0.99) for either of our loci, we report the results of our *BEAST analyses with the strict molecular clock prior.

Three separate MCMC analyses were run for 3×10^8 generations with a 5000 generation burn-in and parameters sampled every 5000 steps. Independent runs were combined using LogCombiner 2.0.2 [34]. Tracer 1.5 (http://beast.bio.ed.ac.uk/Tracer) was used to determine the effective sample size of each parameter and calculate its mean and 95% highest posterior density (95% HPD) interval. Tree topologies were assessed using TreeAnnotator 2.0.2 [34] and visualized in FigTree 1.3.1 (http://tree. bio.ed.ac.uk/software/figtree/).

We used TCS 1.21 [40] to reconstruct allele networks for nuclear loci. Indels were treated as missing data.

Results

Phylogeny of mtDNA haplotypes

Monophyly of both *Calandrella* and *Eremophila* was strongly supported in the phylogenetic analysis of ND2 haplotypes. Both posterior probability values (PP) were equal to 1. The divergence date between these genera was estimated at the Pliocene, 4.9 Ma (95% HPD 3.7–6.4 Ma). *Eremophila* consisted of 11 strongly supported clades (all PPs ≥ 0.99; Fig. 2) diversification of which began with the divergence of Tibetan horned larks (*elwesi*; Fig. 1) from all other *Eremophila* in the Early Pleistocene, 1.4 Ma (1.1–1.8 Ma). The initial divergence of *elwesi* from all other *Eremophia* was followed by a trichotomous split into *bilopha*, south Palearctic alpine (*atlas* and *penicillata* on Fig. 1), and other Palearctic (*flava* and *brandti*) and Nearctic (*alpestris*) clades. This split occurred at the end of the Early Pleistocene, 1 Ma (0.8 – 1.3 Ma). The closer relationship of *bilopha* to all other horned larks than *elwesi* makes currently recognized horned lark paraphyletic in respect to the Temminck's lark.

All Palearctic clades were geographically concordant (Fig. 1). In the addition to *elwesi* and *bilopha*, two Moroccan alpine horned larks (*atlas*) formed a clade which was the sister to a clade composed of west and central Palearctic alpine larks (*balcanica*, *penicillata*, and *albigula*). The second subspecies has a priority over the other two, so we refer to this clade as *penicillata* (Figs. 1, 2, 3, 4, 5). The *atlas* and *penicillata* clades diverged in the Middle Pleistocene 0.286 Ma (0.155–0.410 Ma). The earliest paleontological records for the horned lark from the range of *pennicillata* in the Caucasus are dated at 0.393 ± 0.027 Ma [41].

The last two sister Palearctic clades, *flava* and *brandti* (Fig. 2), were more closely related to Nearctic horned larks than to other Palearctic clades. One of these clades was comprised of north Palearctic birds (*flava*) and the other of birds inhabiting central Palearctic aridlands (*brandti*; Fig. 1). The earliest paleontological records of the horned lark from Europe (southern France and southeastern Germany) that is likely to represent *flava* date to 0.42 ± 0.05 Ma and from northeastern China (likely *brandti*) date to 0.2 – 0.6 Ma.

Divergence of *flava* and *brandti* and of their common ancestor from Nearctic larks dated to the Middle Pleistocene, 0.208 Ma (0.128 – 0.283 Ma) and 0.274 Ma (0.179 – 0.367 Ma), respectively. All Nearctic horned larks were monophyletic (PP = 1; Fig. 2), suggesting a single colonization of the Nearctic by horned larks at the end of Middle Pleistocene, between 0.179 and 0.367 Ma. The horned lark has been recorded in the Bartek Quarry in eastern Nebraska, USA dating to the Middle Pleistocene [42]. Therefore, our estimate of the time of colonization of the Nearctic by the horned lark agrees with the available paleontological data.

Despite a relatively recent history of the horned lark in the Nearctic, the number of strongly supported clades with PP ≥ 0.99 (5) was similar to that in the Palearctic (6). However, none of the three internal nodes connecting Nearctic clades had statistical support (PP ≤ 0.72). Four of the five Nearctic clades had relatively well defined geographic ranges with only limited overlap (Fig 1).

The range of the clade E extended across the Pacific USA from western Washington through western California. All 32 individuals of streaked horned lark (*E. a. stigata*) from western Washington and two from Portland, Oregon shared the same unique ND2 haplotype and are identified by "E*" in Fig. 1. Only a single bird carrying the E* haplotype was found among 17 larks sampled in eastern Washington.

Clade C can be characterized as a Great Basin and southwestern US clade (Fig. 1). C-clade haplotypes dominated samples from south-central Oregon and Nevada. The single samples from southernmost California and central Arizona also belong to this clade. Two of the 19 birds sampled in the Central Valley of California had C-clade haplotypes as well as one bird collected in the Rocky Mountains of central Colorado.

Clade D was widespread across the central part of the contiguous USA from Utah to Wisconsin and Georgia (Fig. 1) and clade A had northeastern Nearctic range and was recorded in Minnesota and New York, USA and Ontario and Newfoundland Is., Canada.

In contrast to other four Nearctic clades, clade B had the largest range and overlapped substantially with all other clades (Fig. 1). Its range extended from northwestern Alaska to Nevada in the southwest, to Georgia in the southeast, and Ontario to the northeast. It was the only clade found among our samples from British Columbia, Canada and from Alaska, alpine and eastern Washington (except a single E* haplotype), Montana, North Dakota, and Minnesota, USA.

Due to extensive geographic overlap of the clade B range with ranges of other Nearctic clades and the monophyly of all Nearctic clades, in the species tree reconstruction we combined all Nearctic birds into a single group identified as *alpestris* for this name has priority among all Nearctic subspecies of the horned lark. The species tree based on ND2 sequences had the same topology and very similar, although slightly younger date estimates for the divergence events (Fig. 2). The biggest difference between ND2 haplotype and species trees was lower PP for all nodes. For two nodes, monophyly of *bilopha* with (*penicillata*, atlas) and ((*flava*, *brandti*), *aplestris*) in respect to *elwesi* and monophyly of *flava* with *brandti* in respect to *aplestris*, the PP probabilities fell below the conventional 0.95 statistical significance cut-off value, from 1 to 0.86 and from 0.98 to 0.6 respectively.

Phylogeny of nuclear haplotypes

Trees based on alleles of nuclear loci strongly supported monophyly of *Eremophila* and *Calandrella* (PP $\geq$ 0.98). The divergence date between the two lark genera was estimated at 2.1 Ma (1.5–2.8 Ma) for ACO1I9 and 2.8 Ma (1.8 – 4.8 Ma) for RHOI1. These estimates were significantly lower than the estimate of 4.9 Ma (95% HPD 3.7–6.4 Ma) based on ND2 sequences.

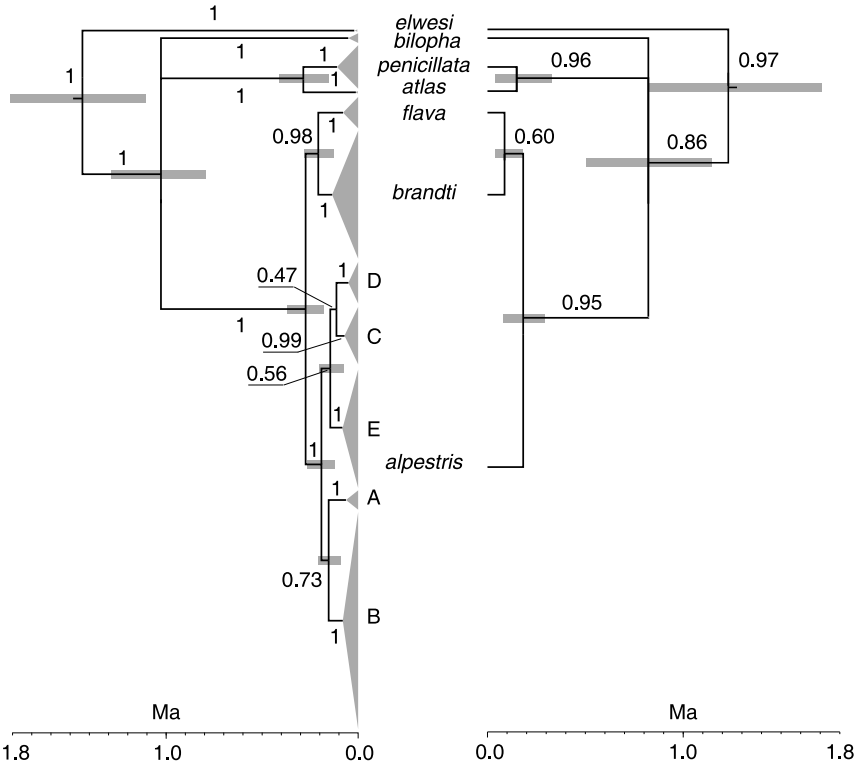

Figure 2. Phylogenetic tree of mtDNA ND2 haplotypes (left) and the species tree based on ND2 sequences (right). Palearctic clades are identified by subspecific names. Nearctic clades are identified by letters (A - E) due to overlap of their ranges. Numbers next to branches show their posterior probability. Gray bars next to nodes indicate their 95% HPD interval for the node age. Scale below each tree indicates time in million years (Ma).
doi:10.1371/journal.pone.0087570.g002

Genetic variation within *Eremophila* appeared poorly structured in both nuclear loci trees (Figs. 3 and 4). Despite the lack of structure, most of ACO1I9 alleles were unique to mtDNA clades. Only three ACO1I9 haplotypes were shared by individuals with mtDNA from different clades. Two of these alleles were shared by individuals with mtDNA from closely related clades (*penicillata* and *atlas*; *alpestris* and *flava*) and one was shared by individuals with distantly related mtDNA clades (*apestris* and *pennicillata* Fig. 3) from distant geographic areas: Kazakhstan, Washington, and Colorado.

In contrast, many RHOI1 alleles were shared by larks carrying mtDNA haplotypes from multiple clades (Fig. 4). The most common RHOI1 allele was shared by individuals from six mtDNA clades on three continents, and another five alleles were found on both northern continents.

The lack of lineage sorting and sharing of alleles had a profound effect on the depth and resolution of the species tree based on nuclear loci. Although the topology of the ACO1I9 species tree was almost identical to that of the ND2 tree (the only exception was the sister relationship of *brandti* and *alpestris* instead of *brandti* and *flava*), only a single node (*penicillata* + *atlas*) had PP $>$ 0.95 whereas all other nodes, including the monophyly of *Eremophila*, had PP $\leq$ 0.74 (Fig. 3). The divergence of *Calandrella* and *Eremophila* was dated to 0.522 Ma (0.214 – 1.675 Ma) and root of *Eremophila* to 0.384 Ma (0.224 – 0.572 Ma). The split between Nearctic *alpestris* and its Palearctic sister *brandti* was dated to 0.120 Ma (0.063 – 0.189 Ma).

In the RHOI1 species tree, the monophyly of *Eremophila* was strongly supported (PP = 1) whereas all other nodes had very low PP values ($0.18 \leq PP \leq 0.54$, Fig. 4). The topology of this tree was similar to that of the ND2 species tree, except *bilopha* appeared to be the sister of *elwesi*, rather than to other horned larks. The divergence of *Calandrella* and *Eremophila* was dated to 0.623 Ma (0.276 – 1.145 Ma) and root of the *Eremophila* to 0.181 Ma (0.105 – 0.276 Ma). The split between Nearctic *alpestris* and its Palearctic sister *brandti* was dated to 0.106 Ma (0.041 – 0.180 Ma).

Multilocus reconstruction of the species tree

The topologies and node support of the species trees based on all three loci and on two nuclear loci were very similar (Fig. 5). The topology of both trees was the same as the topology of the species tree based on RHOI1 (Fig. 4) and differed from ND2 tree topology by positioning *bilopha* as the sister to *elwesi*. Although nodes in the multilocus species trees were much better supported than in the RHOI1 tree, the PP values were $>$ 0.95 only for the monophyly of *Eremophila* and sister relationship between *penicillata* and *atlas*.

Despite the similarity of topologies among single locus and multilocus species trees, the divergence date estimates differed significantly (Table 1). Divergence date estimates were oldest for the ND2 haplotype tree followed closely by the ND2 species tree, whereas the estimates for individual nuclear loci species trees and multilocus species trees were several fold lower.

The comparison of the divergence date estimates of different trees with the available paleontological data suggest that the nuclear species trees and multilocus species trees significantly underestimate divergence dates. For example, the oldest known records of the horned lark from Caucasus are 0.393 $\pm$ 0.027 Ma, Europe – 0.420 $\pm$ 0.050 Ma, northeastern China – 0.2 – 0.6 Ma [41], and from Nebraska - Middle Pleistocene [42] which corresponds to 0.126 – 0.781 Ma. According to the estimates of the 3-loci species tree, the initial divergence within Eremophila is

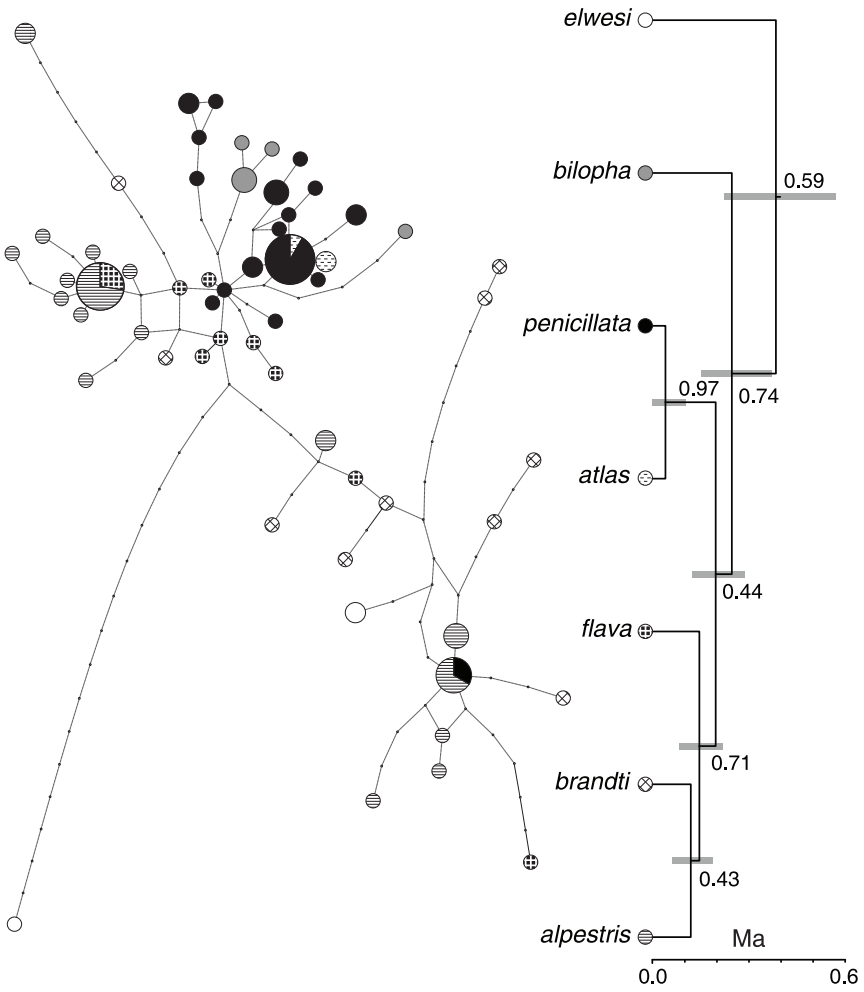

Figure 3. An allele network and species tree based on ACO1I9 sequences. MtDNA clades are identified by names from Fig. 2. Numbers next to branches show their posterior probability. Gray bars next to nodes indicate their 95% HPD interval for the node age. Scale below each tree indicates time in million years (Ma).
doi:10.1371/journal.pone.0087570.g003

younger than the three Palearctic records listed above. Furthermore, only the higher 95% HPD interval limit (0.178 Ma) of the divergence between *alpestris* and the common ancestor of *flava* and *brandti* falls within the Middle Pleistocene, whereas the mean (0.113 Ma) falls within the Late Pleistocene.

In contrast to multilocus species tree, the ND2 haplotype tree produced divergence date estimates consistent with available paleontological data. The earliest record of the horned lark from the Caucasus (0.393 ± 0.027 Ma) is older than the estimate of the divergence date between *pennicillata* and *atlas* 0.286 Ma (95% HPD 0.155 – 0.410 Ma) but younger than the divergence date estimate for the split of the common ancestor of *pennicillata* and *atlas* from other horned lark clades (1.041 Ma; 0.794 – 1.289 Ma). The oldest records from Europe (0.420 ± 0.050 Ma) and northeastern China (0.2 – 0.6 Ma) fall between the *branti/flava* split (0.208 Ma; 0.128 – 0.283 Ma) and divergence of their common ancestor from other horned larks (1.041 Ma; 0.794 – 1.289 Ma). Finally, the divergence of *alpestris* from the common ancestor of *branti* and *flava* 0.274 Ma and its entire 95% HPD interval (0.179 – 0.376 Ma) falls within the Middle Pleistocene (0.126 – 0.781 Ma), the period to which the earliest Nearctic record of the horned lark belongs.

The node ages estimated using our ND2 haplotype and multilocus datasets were strongly correlated. This correlation was not linear and fit the shifted power model: multilocus date = $0.005332 \times (\text{ND2 date} + 2.372115)^{2.89062}$; df = 3, $r^2 = 0.9993$, $P < 0.0001$. Therefore, the difference in divergence date estimates between mtDNA and multilocus data is greatest at the most recent dates then slowly decreases towards the older dates. According to this relationship, both trees will converge to similar date estimates for divergences older than 12 Ma.

Discussion

Performance of individual loci and multilocus analysis

We used one mtDNA, one Z-linked, and one autosomal locus to identify the pattern of geographic structuring of genetic variation within a small lark genus *Eremophila* that consists of two currently recognized species. All three loci individually and their joint analysis support the monophyly of the genus and paraphyly of the horned lark in respect to the Temminck's lark. However, in the reconstruction of the relationships within *Eremophila* the performance of the loci differed significantly.

The tree based on mtDNA ND2 gene identified 11 strongly supported and geographically concordant clades - 6 in the Palearctic and 5 in the Nearctic (Fig. 2). Only Nearctic clades which diversified at the end of the Middle - Late Pleistocene were

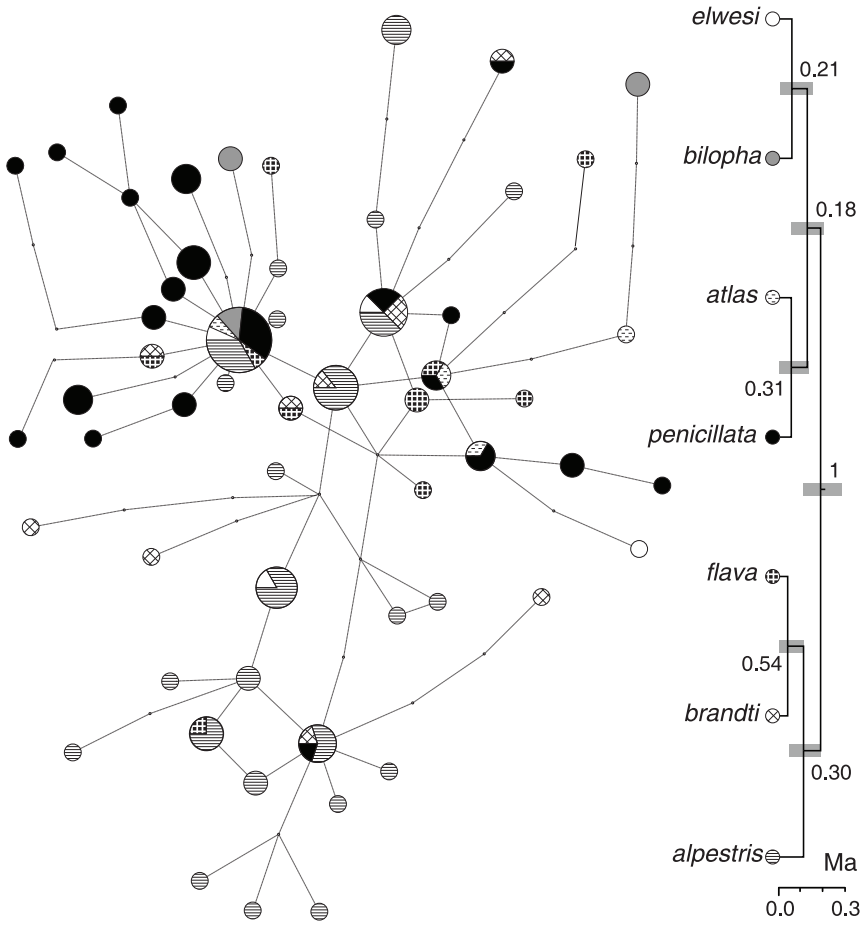

Figure 4. An allele network and species tree based on RHOI1 sequences. MtDNA clades are identified by names from Fig. 2. Numbers next to branches show their posterior probability. Gray bars next to nodes indicate their 95% HPD interval for the node age. Scale below each tree indicates time in million years (Ma).
doi:10.1371/journal.pone.0087570.g004

partially overlapped geographically and relationships among them were weakly supported. Older Palearctic clades did not overlap except, perhaps, *bilopha* and *atlas* in Morocco, where they prefer different habitats (alpine versus rocky deserts, respectively). The relationships among Palearctic clades were well supported with only a single trichotomous split.

The structuring of Palearctic mtDNA clades was not restricted to geography. Old World clades differed in ecology and plumage patterns. These clades could be characterized as inhabiting rocky deserts (*bilopha*), Asian aridlands with sparse grassy and shrubby vegetation (*brandti*), arctic tundra (*flava*), and alpine habitats (*elwesi*, *penicillata*, and *atlas*). Among the latter, *pennicillata* whose range is situated between ranges of distantly related *elwesi* and the closely related sister clade *atlas*, has the most distinct among all *Eremophila* plumage pattern. The black color on the face and upper chest of *penicillata* occupies a much larger area than in other horned larks and is connected, leaving only a small light patch on the throat, whereas in all other forms the black color is not connected and forms separate cheek patches and a bib.

In contrast to mtDNA, nuclear loci provided limited information on structuring of genetic variation within *Eremophila* and provided poor support for the clades identified by mtDNA ND2 sequences. The presence of mtDNA structure concordant with geography, ecology, and plumage patterns and its lack in nuclear or multilocus trees should not be interpreted as disagreement between mtDNA and nuclear loci resulting from the stochastic nature of the coalescence or male-biased dispersal [4,43,44]. Due to differences in the effective population size (N_e) of mtDNA and nuclear loci, the lineage sorting of mtDNA haplotypes requires a quarter of time needed for autosomal loci or a third relative to Z-linked loci. Indeed, the shifted power relationship between divergence date estimates based on ND2 and multi-locus species trees, suggest that for a long time, perhaps as long as 12 Ma, nuclear loci appear to underestimate the age of the events relative to the mtDNA. On the other hand, date estimates based on mtDNA appear to correspond well with the available paleontological data.

In a recent study of rosyfinches (Fringillidae: *Leucosticte*), ACO1I9 and autosomal melanocortin 1 receptor gene (MC1R) failed to identify structuring among species that had a similar level of divergence in the ND2 tree to that of *Eremophila* clades. Species monophyly in *Leucosticte* was strongly supported by maximum likelihood bootstrap values in the mtDNA ND2 tree [44]. In another recent study of the phylogeny of accentors (*Prunellidae*), ACO1I9 performed well identifying lineages that diverged 6 – 3 Ma ago, however, the relationships among lineages younger than 2.5 Ma were unresolved and some species appeared paraphyletic [32]. These and our current findings suggest that nuclear loci may be of limited utility for phylogeographic studies dealing with

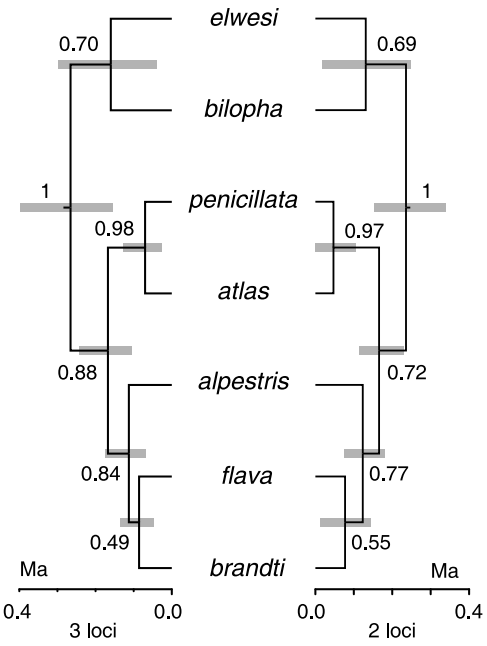

Figure 5. Species trees based on all 3 loci (left) and two nuclear loci (right). Numbers next to branches show their posterior probability. Gray bars next to nodes indicate their 95% HPD interval for the node age. Scale below each tree indicates time in million years (Ma).
doi:10.1371/journal.pone.0087570.g005

lineages evolved in the Middle or Late Pleistocene because they are indeed lagging indicators of divergence events [4].

Combining nuclear loci into a single phylogenetic analysis did not improve phylogenetic resolution. Our multilocus species tree topology was virtually identical to that of the ND2 tree. The only difference was the position of *bilopha* as the sister to *elwesi* in the former and as the sister to all other horned larks in the latter tree. However, only a single of five nodes had statistical support in the species tree, whereas in the ND2 tree, four of these nodes were strongly supported. A number of recent studies using mtDNA and nuclear loci reported similar results - nuclear loci identify phylogeographic structure similar to mtDNA but with poor statistical support if the number of loci is small and increasing with number of loci sampled regardless of whether population divergence was relatively deep [24,32,44–49] or shallow [48,50,51].

Furthermore, our species tree had nearly twice the 95% HPD intervals for node ages relative to their mean value than our ND2 haplotype tree. For the two nodes that had statistical support (PP ≥ 0.98) in both trees, the ancestral node of *Eremophila* and the node connecting *atlas* and *penicillata*, the intervals were 91% and 146% of the mean in the species tree but only 49% and 89% respectively in the ND2 tree. Perhaps, if the group membership is known and a large enough number of nuclear loci is used, they may resolve phylogeographic structure more precisely than a single mtDNA locus.

Systematics implications

All three loci and the multilocus species tree identified paraphyly of the horned lark in respect to the Temminck's lark and therefore, suggest the need for taxonomic revision of *Eremophila*. However, due to the lack of nodal support in our multilocus species tree, we discuss the taxonomic implications of our study in light of the relationships among mtDNA clades identified in our ND2 tree (Fig. 2). Although matrilineal history may not be completely representative of the evolutionary history of *Eremophila* and the relationships among evolutionary units within it, for reasons discussed in the Introduction, we believe that mtDNA is capable of identification of the presence of such units. Furthermore, our estimates of node ages based on the ND2 trees appear to fit the available paleontological data.

According to our mtDNA ND2 tree, the first clade to diverge from the other *Eremophila* was *elwesi*. This clade was composed of only two samples from Tibet in our tree, but it is likely that several other subspecies from central China and Himalayas may belong to this clade as well. For example, in the mtDNA cytochrome-*b* gene tree horned larks from Pakistan were closely related to *elwesi* samples [27].

The initial split of *elwesi* was followed by a trichotomy of *bilopha*, *atlas* + *penicillata*, and the lineage that included *flava*, *brandti*, and all Nearctic larks. The Temminck's lark is already recognized as a distinct species that inhabits rocky deserts inland from the cost of North Africa, Arabia, and Middle East (Fig. 1). The second major lineage combines two closely related, sister clades of high alpine larks. One clade, *atlas*, corresponds to the currently recognized

Table 1. Divergence time estimates and their 95% HPD intervals for selected nodes.

Tree	Date, Ma	Low 95% HPD	High 95% HPD	% of the mean
split of *Eremophila* and *Calandrella*				
ND2 haplotypes	4.936	3.673	6.403	55%
ND2 species	4.528	1.209	6.309	113%
ACO1I9	0.522	0.214	1.675	280%
RHOI1	0.623	0.276	1.145	139%
2 nuclear loci	0.985	0.403	1.961	158%
3 loci	1.674	0.709	2.974	135%
node: root of *Eremophila*				
ND2 haplotypes	1.436	1.108	1.812	49%
ND2	1.257	0.826	1.709	70%
ACO1I9	0.384	0.224	0.572	91%
RHOI1	0.181	0.105	0.276	94%
2 nuclear loci	0.236	0.153	0.341	80%
3 loci	0.266	0.155	0.397	91%
divergence of *alpestris* from Palearctic birds				
ND2 haplotypes	0.274	0.179	0.367	69%
ND2	0.186	0.08	0.291	113%
ACO1I9	0.12	0.063	0.189	105%
RHOI1	0.106	0.041	0.18	131%
2 nuclear loci	0.123	0.075	0.181	86%
3 loci	0.113	0.067	0.174	95%
split of *atlas* and *penicillata*				
ND2 haplotypes	0.286	0.155	0.41	89%
ND2	0.152	0.04	0.327	189%
ACO1I9	0.041	0	0.105	256%
RHOI1	0.052	0	0.131	252%
2 nuclear loci	0.047	0	0.105	223%
3 loci	0.07	0.025	0.127	146%

doi:10.1371/journal.pone.0087570.t001

subspecies *E. a. atlas* inhabiting Atlas mountains of Morocco. Its sister clade, *penicillata*, includes birds inhabiting alpine zone of Palearctic mountains from southeastern Europe to Tian-Shan. In our study this clade was comprised of three subspecies: *E. a. balcanica*, *E. a. penicillata*, and *E. a. albigula*. All these subspecies have black color on the face, neck, and the bib connected, whereas all other larks have a clear separation of the black color between the bib and neck.

The third major, Holarctic lineage was divided into two sister Palearctic and five closely related Nearctic clades. The two Palearctic clades corresponded to subspecies *E. a. flava* breeding in the tundra from Scandinavia to Amguema River in Chukotka and *E. a. brandti* breeding in aridlands from the northwestern border of Kazakhstan to northeastern Inner Mongolia in China.

Although the monophyly of Nearctic clades was strongly supported, the relationships among them were unresolved. Furthermore, breeding range of one clade (B) overlapped with ranges of other clades. Much more detailed sampling of Nearctic horned larks is required to reconstruct clade ranges and elucidate their evolutionary history.

Therefore, our data suggest that all Palearctic clades represent independent evolutionary units and should be treated as distinct species: *elwesi* (although *longirostris* should have priority if it belongs to this clade), *bilopha*, *atlas*, *penicillata*, *brandti*, and *flava*. Nearctic populations should be treated as a single species - *alpestris* pending further investigation.

Conclusions

Phylogeographic studies of Holarctic taxa are challenging due to sample sizes and geographic coverage required for deciphering of even large scale patterns of genetic variation. This is especially true for abundant, wide ranging taxa like *Eremophila* that have extensive phenotypic and ecological variation. Such extensive variation (> 40 described subspecies) prevents a meaningful *a priori* designation of evolutionary units required for analyses of multiple loci with extensive incomplete sorting. Our data show that mtDNA is capable of identifying geographic patterns of genetic variation within and among closely related avian taxa and, ultimately, independent evolutionary units. This power comes from several fold faster lineage sorting and inheritance through females - the further and more frequently dispersing sex in most birds. Our data also show that the intrataxon geographic pattern of mtDNA variation is correlated with the ecological and phenotypic variation and should not be readily dismissed as the result of stochasticity of demographic and evolutionary processes. Conversely, nuclear loci may provide little resolution for the identification of patterns of genetic variation and evolutionary units within abundant, wide-ranging taxa. Perhaps, due to their large effective population size, the lineage sorting of nuclear alleles cannot be completed or significantly advanced between the consecutive recent divergence events. The current study and others, e.g. *Leucosticte* [44], are cases in point. On the other hand, divergent Nearctic lineages within the winter wren identified using only mtDNA [22] were later confirmed to be reproductively isolated [52] and ultimately recognized as distinct species [23].

Thus, we suggest that phylogeographic studies of wide-ranging, abundant birds should place an emphasis on first exploring the geographic variation in mtDNA by sampling a large number of individuals from as many geographic localities as possible. Once this pattern is established, variation in at least a modest number of nuclear and especially Z-linked loci should be explored.

Supporting Information

Appendix S1 Samples used in this study and GenBank accession numbers.
(PDF)

Acknowledgments

We are grateful to the University of Kansas Natural History Museum, University of Washington Burke Museum, US National Museum of Natural History, Natural History Museum University of Oslo, Zoological Museum University of Copenhagen, National History Museum Belgrade, State Darwin Museum, Zoological Museum of Moscow State University, American Museum of Natural History, Yale Peabody Museum, University of Nevada Las Vegas Marjorie Barrick Museum, Louisiana State University Museum of Natural Sciences, University of Minnesota Bell Museum, University of Michigan Museum of Zoology, Royal Ontario Museum, Sandra Cederbaum (University of Georgia), Alaine Camfield and Kathy Martin (University of British Columbia), and Alban Guillaumet (Université Montpellier-2) for sample loans. We are also grateful to Dan Mishmar and two anonymous reviewers for their helpful comments and suggestions for improvement of the original manuscript.

Author Contributions

Conceived and designed the experiments: SVD MR GS IVF YAR. Performed the experiments: SVD MR GS. Analyzed the data: SVD. Contributed reagents/materials/analysis tools: SVD MR GS IVF YAR. Wrote the paper: SVD. Reviewed and edited the initial draft of the manuscript: MR GS IVF YAR.

References

1. Avise JC, Arnold J, Ball RM Jr, Bermingham E, Lamb T, et al. (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics 18: 489–522.
2. Avise JC (2000) Phylogeography; the history and formation of species. Cambridge, Mass.: Harvard University Press. 447 p.
3. Avise JC (2009) Phylogeography: retrospect and prospect. Journal of Biogeography 36: 3–15.
4. Zink RM, Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. Molecular Ecology 17: 2107–2121.
5. Zink RM, Barrowclough GF (2009) Funds enough, and time: mtDNA, nuDNA and the discovery of divergence. Molecular Ecology 18: 1–3.
6. Clarke AL, Sæther BE, Røskaft E (1997) Sex biases in avian dispersal: a reappraisal. Oikos 79: 429–438.
7. Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. Molecular Ecology 13: 729–744.
8. Edwards SV, Kingan SB, Calkins JD, Balakrishnan CN, Jennings WB, et al. (2005) Speciation in birds: Genes, geography, and sexual selection. Proceedings of the National Academy of Sciences of the United States of America 102: 6550–6557.
9. Bazin E, Glemin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. Science 312: 570–572.
10. Edwards S, Bensch S (2009) Looking forwards or looking backwards in avian phylogeography? A comment on Zink and Barrowclough 2008. Molecular Ecology 18: 2930–2933.
11. Krosby M, Rohwer S (2009) A 2000 km genetic wake yields evidence for northern glacial refugia and hybrid zone movement in a pair of songbirds Proceedings of the Royal Society Biological Sciences Series B 276: 615–621.
12. Drovetski SV (2002) Molecular phylogeny of grouse: Individual and combined performance of W-linked, autosomal, and mitochondrial loci. Systematic Biology 51: 930–945.
13. Brito PH, Edwards SV (2009) Multilocus phylogeography and phylogenetics using sequence-based markers. Genetica 135: 439–455.
14. Heled J, Drummond AJ (2010) Bayesian inference of species trees from multilocus data. Molecular Biology and Evolution 27: 570–580.
15. Lee JY, Edwards SV (2008) Divergence across Australia's Carpentarian barrier: statistical phylogeography of the red-backed fairy wren (*Malurus melanocephalus*). Evolution 62: 3117–3134.
16. Hey J, Nielsen R (2004) Multilocus Methods for Estimating Population Sizes, Migration Rates and Divergence Time, With Applications to the Divergence of Drosophila pseudoobscura and D. persimilis. Genetics 167: 747–760.
17. Cramp S (1985) Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic: University Press.

18. Dickinson EC (2003) The Howard & Moore Complete Checklist of the Birds. Princeton, NJ: Princeton University Press.
19. Clements JF (2007) The Clements checklist of birds of the world. Ithaca, NY: Cornell University Press.
20. Beason RC (1995) Horned Lark (*Eremophila alpestris*). In: Poole A, editor. The Birds of North America Online. Ithaca, NY: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online. Available: http://bna.birds.cornell.edu/bna/species/195. Accessed 2013 Nov 28.
21. Stepanyan LS (2003) Conspectus of the ornithological fauna of Russia and adjacent territories (within the borders of the USSR as a historic region). Moscow, Russia: Academkniga. 807 p.
22. Drovetski SV, Zink RM, Rohwer S, Fadeev IV, Nesterov EV, et al. (2004) Complex biogeographic history of a Holarctic passerine. Proceedings of the Royal Society of London, Series B 21: 545–551.
23. Chesser RT, Banks RC, Barker FK, Cicero C, Dunn JL, et al. (2010) Fifty-first supplement to the American Ornithologists' Union Check-List of North American Birds. Auk 127: 726–744.
24. Drovetski SV, Zink RM, Ericson PGP, Fadeev IV (2010) A multi-locus study of pine grosbeak phylogeography supports the pattern of greater intercontinental divergence in Holarctic boreal forest birds compared to birds inhabiting other high-latitude habitats. Journal of Biogeography 37: 696–706.
25. Drovetski SV, Pearson SF, Rohwer S (2005) Streaked horned lark Eremophila alpestris strigata has distinct mitochondrial DNA. Conservation Genetics 6: 875–883.
26. Johnsen A, Rindal E, Ericson PP, Zuccon D, Kerr KR, et al. (2010) DNA barcoding of Scandinavian birds reveals divergent lineages in trans-Atlantic species. Journal of Ornithology 151: 565–578.
27. Alström P, Barnes KN, Olsson U, Barker FK, Bloomer P, et al. (2013) Multilocus phylogeny of the avian family Alaudidae (larks) reveals complex morphological evolution, non-monophyletic genera and hidden species diversity. Molecular Phylogenetics and Evolution.
28. Dove C, Dahlan N, Drovetski S (2013) MtDNA ND2 sequence identifies Streaked Horned Lark (Eremophila alpestris strigata) from birdstrike to US Air Force F-15 at Portland International Airport, Oregon. Conservation Genetics Resources: 1–3.
29. Drovetski SV, Zink RM, Fadeev IV, Nesterov EV, Koblik EA, et al. (2004) Mitochondrial phylogeny of *Locustella* and related genera. Journal of Avian Biology 35: 105–110.
30. Kimball RT, Braun EL, Barker FK, Bowie RCK, Braun MJ, et al. (2009) A well-tested set of primers to amplify regions spread across the avian genome. Molecular Phylogenetics and Evolution 50: 654–660.
31. Primmer CR, Borge T, Lindell J, Sætre GP (2002) Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. Molecular Ecology 11: 603–612.
32. Drovetski SV, Semenov G, Drovetskaya SS, Fadeev IV, Red'kin YA, et al. (2013) Geographic mode of speciation in a mountain specialist Avian family endemic to the Palearctic. Ecology and Evolution.
33. Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. The American Journal of Human Genetics 68: 978–989.
34. Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution.
35. Lerner HRL, Meyer M, James HF, Hofreiter M, Fleischer RC (2011) Multilocus resolution of phylogeny and timescale in the extant adaptive radiation of Hawaiian honeycreepers. Current Biology 21: 1838–1844.
36. Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura RM, editor. Some Mathematical Questions in Biology - DNA Sequence Analysis Providence: American Mathematical Society. pp. 57–86.
37. Yang Z (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. Journal of Molecular Evolution 39: 306–314.
38. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120.
39. Huelsenbeck JP, Crandall KA (1997) Phylogeny estimation and hypothesis testing using maximum likelihood. Annual Review of Ecology and Systematics 28: 437–466.
40. Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. Molecular Ecology 9: 1657–1660.
41. Tyrberg T (1998) Pleistocene birds of the Palearctic: a catalogue.; Paynter Jr. RA, editor. Cambridge, Massachusetts: The Nuttall Ornithological Club. 720 p.
42. Ducey JE (1992) Fossil Birds of the Nebraska Region. Transactions of the Nebraska Academy of Sciences 19: 83–96.
43. McKay BD, Zink RM (2009) The causes of mitochondrial DNA gene tree paraphyly in birds. Molecular Phylogenetics and Evolution.
44. Drovetski SV, Zink RM, Mode NA (2009) Patchy distributions belie morphological and genetic homogeneity in rosy-finches. Molecular Phylogenetics and Evolution 50: 437–445.
45. Peters JL, McCracken KG, Pruett CL, Rohwer S, Drovetski SV, et al. (2012) A parapatric propensity for breeding precludes the completion of speciation in common teal (Anas crecca, sensu lato). Molecular Ecology 21: 4563–4577.
46. Smith BT, Klicka J (2013) Examining the Role of Effective Population Size on Mitochondrial and Multilocus Divergence Time Discordance in a Songbird. PLoS ONE 8: e55161.
47. Hung C-M, Drovetski SV, Zink RM (2012) Multilocus coalescence analyses support a mtDNA-based phylogeographic historyfor a widespread Palearctic passerine bird, Sitta europaea. Evolution 66: 2850–2864.
48. Arbeláez-Cortés E, Milá B, Navarro-Sigüenza AG (2014) Multilocus analysis of intraspecific differentiation in three endemic bird species from the northern Neotropical dry forest. Molecular Phylogenetics and Evolution 70: 362–377.
49. Pavlova A, Zink RM, Drovetski SV, Rohwer S (2008) Pleistocene evolution of closely related sand martins Riparia riparia and R. diluta. Molecular Phylogenetics and Evolution 48: 61–73.
50. Hung C-M, Drovetski S, Zink R (2013) Multilocus test of the absence of mtDNA phylogeographic structure in a widespread wader, the Common Sandpiper (Actitis hypoleucos). Journal of Ornithology 154: 1105–1113.
51. Peters JL, Zhuravlev YN, Fefelov I, Humphries EM, Omland KE (2008) Multilocus phylogeography of a Holarctic duck: colonization of North America from Eurasia by gadwall (Anas strpera). Evolution 62: 1469–1483.
52. Toews DPL, Irwin DE (2008) Cryptic speciation in a Holarctic passerine revealed by genetic and bioacoustic analyses. Molecular Ecology 17: 2691–2705.
53. de Juana E, Suárez F, Ryan P, Alström P, Donald P (2004) Family Alaudidae (Larks). In: del Hoyo J, Elliott A, Christie D, editors. Handbook of the birds of the world, Volume 9: Cotingas to Pipits and Wagtails. Barcelona: Lynx Edicions. pp. 496–601.
54. Mullarney K, Svensson L, Zetterström D, Grant PJ (2006) Bird guide. The most complete field guide to the birds of Britain and Europe. London: HarperCollins Publishers Ltd.

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
SVD2112	UWBM 64797	*Calandrella brachydactyla*	*Calandrella*	M	14-Jul-99	43.90	40.78	KF735312	KF735211, KF735212	KF735505, KF735506
CDS4814	UWBM 46251	*Calandrella brachydactyla*	*Calandrella*	F	19-May-93	43.83	76.35	KF735311		
SVD1138	UWBM 56909	*Calandrella brachydactyla*	*Calandrella*	F	2-Jun-96	46.22	47.77	KF735313	KF735213	KF735507, KF735508
NHMUO17026	NHMUO 17026	*Eremophila alpestris*	*elwesi*		15-Jul-06	30.26	90.66	KF735315	KF735214, KF735215	KF735509, KF735510
NHMUO17038	NHMUO 17038	*Eremophila alpestris*	*elwesi*		14-Jul-06	30.74	90.95	KF735314	KF735216	KF735511, KF735512
AGB1	U. de Montpellier	*Eremophila bilopha*	*bilopha*	M		34.03	353.15	KF735318	KF735217, KF735218	KF735527, KF735528
AGB2	U. de Montpellier	*Eremophila bilopha*	*bilopha*	M		34.03	353.15	KF735319	KF735219, KF735220	KF735531, KF735532
AGB3	U. de Montpellier	*Eremophila bilopha*	*bilopha*			34.03	353.15	KF735320	KF735221	KF735533, KF735534
ZMUC135714	ZMUC 135714	*Eremophila bilopha*	*bilopha*			36.78	10.17	KF735316		
ZMUC135715	ZMUC 135715	*Eremophila bilopha*	*bilopha*			36.78	10.17	KF735317	KF735222	
AGA1	U. de Montpellier	*Eremophila alpestris*	*atlas*	M		34.03	353.15	KF735321	KF735223, KF735224	KF735525, KF735526
AGA2	U. de Montpellier	*Eremophila alpestris*	*atlas*			34.03	353.15	KF735322	KF735225	KF735529, KF735530
MR1061	NHMB	*Eremophila alpestris*	*penicillata*	M	5-Aug-10	43.31	22.86	KF735442	KF735226, KF735227	KF735583, KF735584
MR1062	NHMB	*Eremophila alpestris*	*penicillata*	M	5-Aug-10	43.31	22.86	KF735443	KF735228, KF735229	KF735585, KF735586
MR0950	NHMB	*Eremophila alpestris*	*penicillata*	M	18-Jun-10	40.47	44.19	KF735444	KF735230, KF735231	KF735575, KF735576
MR0951	NHMB	*Eremophila alpestris*	*penicillata*	M	18-Jun-10	40.47	44.19	KF735445	KF735232, KF735233	KF735577, KF735578
MR0952	NHMB	*Eremophila alpestris*	*penicillata*	M	18-Jun-10	40.47	44.19	KF735446	KF735234, KF735235	KF735579, KF735580
MR0953	NHMB	*Eremophila alpestris*	*penicillata*	M	18-Jun-10	40.47	44.19	KF735447	KF735236, KF735237	KF735581, KF735582
IVF1021	SDM	*Eremophila alpestris*	*penicillata*	M	25-May-11	40.43	44.23	KF735331	KF735238, KF735239	KF735571, KF735572
IVF1100	SDM	*Eremophila alpestris*	*penicillata*	F	16-Jun-11	40.42	44.25	KF735332	KF735240	KF735573, KF735574
SVD4632	SDM	*Eremophila alpestris*	*penicillata*	M	25-May-11	40.43	44.23	KF735486	KF735241, KF735242	KF735623, KF735624
SVD4633	SDM	*Eremophila alpestris*	*penicillata*	M	25-May-11	40.43	44.23	KF735487	KF735243, KF735244	KF735625, KF735626
EAK192	MSUZM	*Eremophila alpestris*	*penicillata*	M	18-May-05	35.99	51.63	KF735323	KF735245, KF735246	KF735543, KF735544
EAK193	MSUZM	*Eremophila alpestris*	*penicillata*	M	18-May-05	35.99	51.63	KF735324	KF735247, KF735248	KF735545, KF735546
EAK194	MSUZM	*Eremophila alpestris*	*penicillata*	M	18-May-05	35.99	51.63	KF735325	KF735249, KF735250	KF735547, KF735548
EAK195	MSUZM	*Eremophila alpestris*	*penicillata*	M	18-May-05	35.99	51.63	KF735326		KF735549, KF735550
EAK196	MSUZM	*Eremophila alpestris*	*penicillata*	M	18-May-05	35.99	51.63	KF735327		KF735551, KF735552
GAV167	UWBM 46543	*Eremophila alpestris*	*penicillata*	M	24-May-93	42.98	75.88	KF735328	KF735251, KF735252	KF735555, KF735556
GAV168	UWBM 46544	*Eremophila alpestris*	*penicillata*	F	24-May-93	42.98	75.88	KF735329		
GAV169	UWBM 46545	*Eremophila alpestris*	*penicillata*	M	24-May-93	42.98	75.88	KF735330	KF735253, KF735254	KF735557, KF735558
F091	MSUZM	*Eremophila alpestris*	*penicillata*	M	11-May-10	42.95	80.00		KF735255, KF735256	KF735553, KF735554
IVYBN	MSUZM	*Eremophila alpestris*	*penicillata*	M	11-May-10	42.95	80.00	KF735333	KF735257, KF735258	KF735521, KF735522
NHMUO18830	NHMUO 18830	*Eremophila alpestris*	*flava*	M	19-Jun-06	61.42	8.87	KF735334	KF735259, KF735260	KF735513, KF735514
ZMUC123699	ZMUC 123699	*Eremophila alpestris*	*flava*			55.68	12.57	KF735504		
NHMUO8534	NHMUO 8534	*Eremophila alpestris*	*flava*		17-Jul-05	70.48	28.91	KF735343	KF735261, KF735262	
NHMUO30995	NHMUO 30995	*Eremophila alpestris*	*flava*	M	24-Jun-10	70.39	31.01	KF735340	KF735263, KF735264	KF735517, KF735518

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
NHMUO8506	NHMUO 8506	*Eremophila alpestris*	*flava*		8-Jul-04	70.35	31.02	KF735342	KF735265	KF735523, KF735524
NHMUO31024	NHMUO 31024	*Eremophila alpestris*	*flava*	M	27-Jun-10	70.34	31.03	KF735341	KF735266, KF735267	KF735519, KF735520
MVK139	UWBM 74056	*Eremophila alpestris*	*flava*	F	18-May-01	59.83	52.72	KF735460	KF735268	KF735587, KF735588
MVK140	UWBM 74057	*Eremophila alpestris*	*flava*	F	18-May-01	59.83	52.72	KF735461		
MVK141	UWBM 74058	*Eremophila alpestris*	*flava*	F	18-May-01	59.83	52.72	KF735462		
SVD1431	UWBM 59593	*Eremophila alpestris*	*flava*	M	12-Jun-97	68.02	68.60	KF735470		
SVD1432	UWBM 59594	*Eremophila alpestris*	*flava*	F	12-Jun-97	68.02	68.60	KF735471		
SVD1433	UWBM 59595	*Eremophila alpestris*	*flava*	F	12-Jun-97	68.02	68.60	KF735472		
N85	MSUZM	*Eremophila alpestris*	*flava*		9-Aug-03	72.83	105.83	KF735463	KF735269	KF735589, KF735590
NAM173	UWBM 66344	*Eremophila alpestris*	*brandti*	M	19-Jun-00	50.38	89.87	KF735468		
MSUZM1792000	UWBM 75737	*Eremophila alpestris*	*brandti*	M	5-Jun-00	50.15	90.14	KF735451		
MSUZM1802000	UWBM 75738	*Eremophila alpestris*	*brandti*	M	5-Jun-00	50.15	90.14	KF735452		
MSUZM1812000	UWBM 75739	*Eremophila alpestris*	*brandti*	M	5-Jun-00	50.15	90.14	KF735453		
MSUZM1822000	UWBM 75740	*Eremophila alpestris*	*brandti*	M	5-Jun-00	50.15	90.14	KF735454		
MSUZM2242000	UWBM 75779	*Eremophila alpestris*	*brandti*	M	8-Jun-00	50.15	90.14	KF735455		
NAM161	UWBM 66332	*Eremophila alpestris*	*brandti*	F	17-Jun-00	50.35	90.48	KF735466		
NAM162	UWBM 66333	*Eremophila alpestris*	*brandti*	M	17-Jun-00	50.35	90.48	KF735467		
MSUZM1062000	UWBM 75668	*Eremophila alpestris*	*brandti*	M	27-May-00	50.62	91.52	KF735448		
MSUZM1242000	UWBM 75684	*Eremophila alpestris*	*brandti*	M	31-May-00	50.28	90.66	KF735449		
MSUZM1252000	UWBM 75685	*Eremophila alpestris*	*brandti*	M	31-May-00	50.28	90.66	KF735450		
NAM140	UWBM 66312	*Eremophila alpestris*	*brandti*	M	13-Jun-00	50.65	91.60	KF735464		
NAM142	UWBM 66314	*Eremophila alpestris*	*brandti*	F	13-Jun-00	50.65	91.60	KF735465		
MSUZMn0601999	UWBM 78960	*Eremophila alpestris*	*brandti*	M	29-May-99	51.35	94.55	KF735457		
MSUZMn0561999	UWBM 67579	*Eremophila alpestris*	*brandti*	F	28-May-99	51.42	94.77	KF735456		
MSUZMp0511999	UWBM 67679	*Eremophila alpestris*	*brandti*	M	6-Jun-99	50.03	95.03	KF735459		
RCF1976	UWBM 66603	*Eremophila alpestris*	*brandti*	M	9-Jul-00	50.07	95.13	KF735469		
MSUZMn2381999	UWBM 71026	*Eremophila alpestris*	*brandti*	M	29-Jun-99	50.28	96.40	KF735458		
DAB2299	UWBM 58019	*Eremophila alpestris*	*brandti*	M	11-Jun-97	48.12	100.37	KF735366		
B10281	USNM 586726	*Eremophila alpestris*	*brandti*		6-May-97	44.90	100.57	KF735347		
BKS3990	UWBM 57873	*Eremophila alpestris*	*brandti*	M	5-Jun-97	44.90	100.57	KF735353		
DAB2261	UWBM 57984	*Eremophila alpestris*	*brandti*	F	5-Jun-97	44.90	100.57	KF735364		
DAB2262	UWBM 57985	*Eremophila alpestris*	*brandti*	F	5-Jun-97	44.90	100.57	KF735365		
B10187	USNM 586670	*Eremophila alpestris*	*brandti*		28-May-97	43.36	103.18	KF735345		
B10275	USNM 586720	*Eremophila alpestris*	*brandti*		28-May-97	43.36	103.18	KF735346		
DAB2222	UWBM 57949	*Eremophila alpestris*	*brandti*	F	28-May-97	43.37	103.18	KF735362		
DAB2234	UWBM 57960	*Eremophila alpestris*	*brandti*	M	29-May-97	43.37	103.18	KF735363		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
LMC9745	AMNH	*Eremophila alpestris*	*brandti*		14-Jul-93	43.36	103.18	KF735439		
B10177	USNM 621499	*Eremophila alpestris*	*brandti*		24-May-97	47.33	105.40	KF735344		
CDS4894	UWBM 46334	*Eremophila alpestris*	*brandti*	F	16-Jun-93	51.57	106.85	KF735354	KF735270	KF735535, KF735536
CDS4895	UWBM 46335	*Eremophila alpestris*	*brandti*	M	16-Jun-93	51.57	106.85	KF735355	KF735271, KF735272	KF735537, KF735538
CDS4913	UWBM 46353	*Eremophila alpestris*	*brandti*	M	18-Jun-93	51.57	106.85	KF735356		
DAB2584	UWBM 60021	*Eremophila alpestris*	*brandti*	F	9-May-98	47.77	112.15	KF735367		
DAB2600	UWBM 60037	*Eremophila alpestris*	*brandti*	F	11-May-98	48.03	114.38	KF735368		
DAB2602	UWBM 60039	*Eremophila alpestris*	*brandti*	F	11-May-98	48.03	114.38	KF735369		
DAB2603	UWBM 60040	*Eremophila alpestris*	*brandti*	F	12-May-98	48.03	114.38	KF735370		
DAB2604	UWBM 60041	*Eremophila alpestris*	*brandti*	M	12-May-98	48.03	114.38	KF735371		
IUK2404	USNM 640019	*Eremophila alpestris*	*brandti*	F	28-Jun-05	50.50	115.00	KF735419		
IUK2405	USNM 640020	*Eremophila alpestris*	*brandti*	M	28-Jun-05	50.50	115.00	KF735420		
IUK2406	USNM 640021	*Eremophila alpestris*	*brandti*	F	28-Jun-05	50.50	115.00	KF735421		
IUK2413	YPM	*Eremophila alpestris*	*brandti*	F	28-Jun-05	50.50	115.00	KF735422		
IUK2414	USNM 640028	*Eremophila alpestris*	*brandti*	M	28-Jun-05	50.50	115.00	KF735423		
IUK2423	YPM	*Eremophila alpestris*	*brandti*	M	30-Jun-05	50.50	115.00	KF735424		
IUK2426	USNM 640037	*Eremophila alpestris*	*brandti*	M	30-Jun-05	50.50	115.00	KF735425		
IUK2427	USNM 640038	*Eremophila alpestris*	*brandti*	M	30-Jun-05	50.50	115.00	KF735426		
IVF0687	SDM	*Eremophila alpestris*	*brandti*	F	30-Jun-05	50.50	115.00	KF735427		
IVF0688	SDM	*Eremophila alpestris*	*brandti*	M	30-Jun-05	50.50	115.00	KF735428		
SVD3556	USNM 640648	*Eremophila alpestris*	*brandti*	M	28-Jun-05	50.50	115.00	KF735480	KF735273, KF735274	
SVD3568	USNM 640660	*Eremophila alpestris*	*brandti*	F	30-Jun-05	50.50	115.00	KF735481	KF735275	KF735613, KF735614
SVD3569	USNM 640661	*Eremophila alpestris*	*brandti*	M	30-Jun-05	50.50	115.00	KF735482	KF735276, KF735277	KF735615, KF735616
SVD3570	USNM 640662	*Eremophila alpestris*	*brandti*	M	30-Jun-05	50.50	115.00	KF735483	KF735278, KF735279	KF735617, KF735618
CSW5801	UWBM 59834	*Eremophila alpestris*	*brandti*	F	15-May-98	48.43	115.10	KF735357		
CSW5803	UWBM 59836	*Eremophila alpestris*	*brandti*	M	15-May-98	48.43	115.10	KF735358		
SVD2365	UWBM 66901	*Eremophila alpestris*	*alpestris*	M	2-Jun-00	65.73	194.14	DQ187408		
SVD2366	UWBM 66902	*Eremophila alpestris*	*alpestris*	M	2-Jun-00	65.66	193.82	DQ187409		
SVD2367	UWBM 66903	*Eremophila alpestris*	*alpestris*	M	3-Jun-00	65.97	193.82	DQ187410		
SVD2368	UWBM 66904	*Eremophila alpestris*	*alpestris*	F	3-Jun-00	65.97	193.82	DQ187411		
SVD2369	UWBM 66905	*Eremophila alpestris*	*alpestris*	M	6-Jun-00	65.43	195.23	DQ187412		
UAMX014	UAM 7608	*Eremophila alpestris*	*alpestris*	F	24-May-97	64.84	212.28	DQ187390		
B13413	USNM 622679	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.58	213.25	DQ187391		
B13421	USNM 622678	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.58	213.25	DQ187392		
B13422	USNM 622677	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.58	213.25	DQ187393		
B13492	USNM 601741	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.58	213.25	DQ187397		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
B13493	USNM 601742	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.58	213.25	DQ187398		
B13425	USNM 622682	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.63	213.39	DQ187394		
B13426	USNM 622681	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.63	213.39	DQ187395		
B13485	USNM 601740	*Eremophila alpestris*	*alpestris*		22-Aug-00	64.64	213.40	DQ187396		
KSW1478	UAM 7752	*Eremophila alpestris*	*alpestris*	F	24-Jun-97	65.48	214.60	DQ187399		
KSW1479	UAM 7617	*Eremophila alpestris*	*alpestris*	M	24-Jun-97	65.48	214.60	DQ187400		
KSW1485	UAM 8957	*Eremophila alpestris*	*alpestris*	F	24-Jun-97	65.48	214.60	DQ187401		
KSW1500	UAM 7607	*Eremophila alpestris*	*alpestris*	F	25-Jun-97	65.48	214.60	DQ187402		
UAMX775	UAM 10087	*Eremophila alpestris*	*alpestris*	M	25-Jun-97	65.48	214.60	DQ187403		
UAMX790	UAM 10088	*Eremophila alpestris*	*alpestris*	M	25-Jun-97	65.48	214.60	DQ187404		
DAB0686	UWBM 53941	*Eremophila alpestris*	*alpestris*	M	22-Jun-95	65.50	214.62	DQ187405		
DAB0688	UWBM 53943	*Eremophila alpestris*	*alpestris*	F	22-Jun-95	65.50	214.62	DQ187406		
DAB0689	UWBM 53944	*Eremophila alpestris*	*alpestris*	F	22-Jun-95	65.50	214.62	DQ187407		
SVD3377	USNM 640482	*Eremophila alpestris*	*alpestris*	F	31-Jul-04	63.06	214.24			KF735611, KF735612
SVD4092	YPM	*Eremophila alpestris*	*alpestris*	M	6-Jul-07	64.59	218.73	KF735484	KF735280, KF735281	KF735619, KF735620
SVD4093	YPM	*Eremophila alpestris*	*alpestris*	M	6-Jul-07	64.59	218.73	KF735485	KF735282, KF735283	KF735621, KF735622
UBC28334	UBC28334	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735491		
UBC29857	UBC29857	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735492		
UBC29858	UBC29858	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735493		
UBC29860	UBC29860	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735494		
UBC29861	UBC29861	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735495		
UBC36201	UBC36201	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735496		
UBC36202	UBC36202	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735497		
UBC36206	UBC36206	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735498		
UBC36209	UBC36209	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735499		
UBC36218	UBC36218	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735500		
UBC36242	UBC36242	*Eremophila alpestris*	*alpestris*		nestling feather	54.78	232.72	KF735501	KF735284	KF735627, KF735628
SMB413	UWBM	*Eremophila alpestris*	*alpestris*			46.80	235.90	DQ187457	KF735285	KF735599, KF735600
SMB414	UWBM	*Eremophila alpestris*	*alpestris*			46.80	235.90	DQ187458		
SMB415	UWBM	*Eremophila alpestris*	*alpestris*			46.80	235.90	DQ187459		
SMB416	UWBM	*Eremophila alpestris*	*alpestris*			46.80	235.90	DQ187460		
SMB417	UWBM	*Eremophila alpestris*	*alpestris*			46.80	235.90	DQ187462		
SMB418	UWBM	*Eremophila alpestris*	*alpestris*			46.10	236.70	DQ187463		
SMB419	UWBM	*Eremophila alpestris*	*alpestris*			46.10	236.70	DQ187464		
SMB420	UWBM	*Eremophila alpestris*	*alpestris*			46.10	236.70	DQ187465		
SMB421	UWBM	*Eremophila alpestris*	*alpestris*			46.10	236.70	DQ187466		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
SMB422	UWBM	*Eremophila alpestris*	*alpestris*			46.10	236.70	DQ187467		
JMB758	UWBM 46848	*Eremophila alpestris*	*alpestris*	F	11-Aug-91	40.18	237.77	KF735438		
RCF2596	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187461		
SMB397	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187444		
SMB398	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187445		
SMB401	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187446		
SMB402	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187447		
SMB403	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187448		
SMB404	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187449		
SMB405	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187450		
SMB406	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187451		
SMB407	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187452		
SMB408	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187453		
SMB410	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187454		
SMB411	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187455		
SMB412	UWBM	*Eremophila alpestris*	*alpestris*			47.10	237.40	DQ187456		
EVL481	UWBM 77057	*Eremophila alpestris*	*alpestris*		21-May-02	47.09	237.42	DQ187437		
EVL483	UWBM 77059	*Eremophila alpestris*	*alpestris*		2-Jul-02	47.09	237.42	DQ187439		
EVL484	UWBM 77060	*Eremophila alpestris*	*alpestris*		2-Jul-02	47.09	237.42	DQ187440		
GKD375	UWBM 77062	*Eremophila alpestris*	*alpestris*		3-Jul-02	47.09	237.42	DQ187441		
GKD377	UWBM 77064	*Eremophila alpestris*	*alpestris*		17-Jul-02	47.09	237.42	DQ187443		
EVL482	UWBM 77058	*Eremophila alpestris*	*alpestris*		16-Jul-02	46.97	237.51	DQ187438		
GKD376	UWBM 77063	*Eremophila alpestris*	*alpestris*		25-Jun-02	46.97	237.51	DQ187442		
EVL485	UWBM 77061	*Eremophila alpestris*	*alpestris*	F	9-May-02	47.14	237.52	KF735395		
SVD0999	UWBM 53445	*Eremophila alpestris*	*alpestris*	M	19-Jul-95	48.77	238.08	DQ187484	KF735286, KF735287	KF735601, KF735602
SVD1000	UWBM 53410	*Eremophila alpestris*	*alpestris*	F	19-Jul-95	48.77	238.08	DQ187485		
SVD1001	UWBM 53411	*Eremophila alpestris*	*alpestris*	M	19-Jul-95	48.77	238.08	DQ187486		
NKJ5923	MVZ 171608	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187413		
NKJ5924	MVZ 171609	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187414		
NKJ5925	MVZ 171610	*Eremophila alpestris*	*alpestris*	M	30-Mar-88	37.63	238.53	DQ187415	KF735288, KF735289	
NKJ5926	MVZ 171611	*Eremophila alpestris*	*alpestris*	M	30-Mar-88	37.63	238.53	DQ187416	KF735290, KF735291	KF735591, KF735592
NKJ5927	MVZ 171612	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187417		
NKJ5928	MVZ 171613	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187418	KF735292, KF735293	
NKJ5929	MVZ 171614	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187419		
NKJ5930	MVZ 171615	*Eremophila alpestris*	*alpestris*	F	30-Mar-88	37.63	238.53	DQ187420	KF735294	KF735593, KF735594
NKJ5931	MVZ 171616	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187421		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
NKJ5932	MVZ 171617	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187422		
NKJ5933	MVZ 171618	*Eremophila alpestris*	*alpestris*		30-Mar-88	37.63	238.53	DQ187423		
NKJ6003	MVZ 171619	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187424		
NKJ6004	MVZ 171620	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187425		
NKJ6005	MVZ 171621	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187426		
NKJ6006	MVZ 171622	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187427		
NKJ6007	MVZ 171623	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187428		
NKJ6008	MVZ 171624	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187429		
NKJ6009	MVZ 171625	*Eremophila alpestris*	*alpestris*		16-May-88	37.63	238.53	DQ187430		
NKJ6010	MVZ 171626	*Eremophila alpestris*	*alpestris*	F	16-May-88	37.63	238.53	DQ187431	KF735295	KF735595, KF735596
SVD2356	UWBM 66190	*Eremophila alpestris*	*alpestris*	M	16-Jun-00	42.48	239.72	DQ187432	KF735296, KF735297	KF735603, KF735604
SVD2357	UWBM 66191	*Eremophila alpestris*	*alpestris*	M	16-Jun-00	42.48	239.72	DQ187433		
SVD2358	UWBM 66194	*Eremophila alpestris*	*alpestris*	M	16-Jun-00	42.48	239.72	DQ187434		
SVD2359	UWBM 66192	*Eremophila alpestris*	*alpestris*	M	16-Jun-00	42.48	239.72	DQ187435		
SVD2360	UWBM 66193	*Eremophila alpestris*	*alpestris*	M	16-Jun-00	42.48	239.72	DQ187436		
SVD2207	UWBM 64947	*Eremophila alpestris*	*alpestris*	M	28-Apr-00	47.02	239.98	DQ187483		
SAR7267	UWBM 58576	*Eremophila alpestris*	*alpestris*	F	19-Jul-97	48.67	240.07	DQ187487		
DAB0411	UWBM 51057	*Eremophila alpestris*	*alpestris*	M	12-Mar-94	47.81	240.36	DQ187477		
CSW6422	UWBM 74049	*Eremophila alpestris*	*alpestris*	M	25-Apr-03	47.05	240.48	DQ187475		
CSW6423	UWBM 74050	*Eremophila alpestris*	*alpestris*	M	25-Apr-03	47.05	240.48	DQ187476		
CSW5717	UWBM 58529	*Eremophila alpestris*	*alpestris*	M	18-Jul-97	48.68	240.49	DQ187473		
CSW5718	UWBM 58530	*Eremophila alpestris*	*alpestris*		18-Jul-97	48.68	240.49	DQ187474		
PLG216	UWBM 58564	*Eremophila alpestris*	*alpestris*	M	18-Jul-97	48.68	240.49	DQ187478	KF735298, KF735299	KF735597, KF735598
SVD2197	UWBM 64941	*Eremophila alpestris*	*alpestris*	M	15-Apr-00	47.00	240.57	DQ187479		
SVD2198	UWBM 64942	*Eremophila alpestris*	*alpestris*	M	15-Apr-00	47.00	240.57	DQ187480		
SVD2199	UWBM 64943	*Eremophila alpestris*	*alpestris*	M	15-Apr-00	47.00	240.57	DQ187481		
SVD2200	UWBM 64944	*Eremophila alpestris*	*alpestris*	F	15-Apr-00	47.00	240.57	DQ187482		
CSW5140a	UWBM 52515	*Eremophila alpestris*	*alpestris*	F	18-Apr-95	47.61	240.71	DQ187471		
CSW5141a	UWBM 52516	*Eremophila alpestris*	*alpestris*	M	18-Apr-95	47.61	240.71	DQ187472		
BKS1405	UWBM 69686	*Eremophila alpestris*	*alpestris*	F	12-Mar-94	46.93	240.82	DQ187468		
BKS1417	UWBM 69698	*Eremophila alpestris*	*alpestris*	M	11-Mar-94	46.93	240.82	DQ187469		
BKS1419	UWBM 69700	*Eremophila alpestris*	*alpestris*	M	13-Mar-94	46.93	240.82	DQ187470		
JK00336	MBM 8470	*Eremophila alpestris*	*alpestris*		21-Jun-00	37.28	243.47	KF735431		
DHB3640	MBM 8472	*Eremophila alpestris*	*alpestris*		21-Jul-00	37.18	243.90	KF735378		
DHB3642	MBM 8474	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.18	243.90	KF735379		
DHB3643	MBM 8473	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.18	243.90	KF735380		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
DHB3644	MBM 8471	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.18	243.90	KF735381		
DHB3646	MBM 8478	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.18	243.90	KF735382		
DHB3647	MBM 8477	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.18	243.90	KF735383		
DHB3648	MBM 8481	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.18	243.90	KF735384		
DHB3649	MBM 8479	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.08	243.90	KF735385		
DHB3650	MBM 8480	*Eremophila alpestris*	*alpestris*		8-Jun-00	37.08	243.90	KF735386		
DHB3982	MBM 9771	*Eremophila alpestris*	*alpestris*		22-May-01	37.18	243.92	KF735387		
DHB3992	MBM 9772	*Eremophila alpestris*	*alpestris*		22-May-01	37.18	243.92	KF735388		
DHB4005	MBM 9773	*Eremophila alpestris*	*alpestris*		22-May-01	37.18	243.92	KF735389		
JK00346	MBM 8504	*Eremophila alpestris*	*alpestris*		7-Jun-00	37.18	243.93	KF735432		
JK00348	MBM 8505	*Eremophila alpestris*	*alpestris*		7-Jun-00	37.18	243.93	KF735433		
GAV1938	MBM 8546	*Eremophila alpestris*	*alpestris*		12-Jul-00	37.12	243.95	KF735399		
GAV1939	MBM 8547	*Eremophila alpestris*	*alpestris*		12-Jul-00	37.12	243.95	KF735400		KF735561, KF735562
GAV1940	MBM 8548	*Eremophila alpestris*	*alpestris*		12-Jul-00	37.12	243.95	KF735401		
JK00354	MBM 8503	*Eremophila alpestris*	*alpestris*		12-Jun-00	37.12	243.95	KF735434		
GAV1937	MBM 8545	*Eremophila alpestris*	*alpestris*		7-Jun-00	37.12	244.00	KF735398		
B24753	LSUMNS	*Eremophila alpestris*	*alpestris*		7-May-96	33.13	244.49	KF735349		
DHB2538	MBM 5676	*Eremophila alpestris*	*alpestris*	M	11-Jul-98	37.68	246.94	KF735377	KF735300, KF735301	KF735541, KF735542
JDW0038	BMUM 43727	*Eremophila alpestris*	*alpestris*		18-Jun-94	46.60	247.05	KF735429		
B7308	USNM	*Eremophila alpestris*	*alpestris*			33.53	247.63	KF735352		
X7327	BMUM 43726	*Eremophila alpestris*	*alpestris*	M	19-Jun-94	48.51	249.03	KF735502		
JK9477	BMUM 43724	*Eremophila alpestris*	*alpestris*	M	20-Jun-94	48.57	249.70	KF735435		
JK9478	BMUM 43730	*Eremophila alpestris*	*alpestris*	M	20-Jun-94	48.57	249.70	KF735436		
JK9479	BMUM 43728	*Eremophila alpestris*	*alpestris*	F	20-Jun-94	48.57	249.70	KF735437		
X7328	BMUM 43725	*Eremophila alpestris*	*alpestris*	M	20-Jun-94	48.65	249.90	KF735503		
GAV0861	UWBM 56362	*Eremophila alpestris*	*alpestris*	M	24-Jun-96	38.69	253.52	KF735403	KF735302, KF735303	KF735559, KF735560
DHB1994	UWBM 70312	*Eremophila alpestris*	*alpestris*	F	21-Jun-96	39.60	254.29	KF735372		
DHB1995	UWBM 70313	*Eremophila alpestris*	*alpestris*	M	21-Jun-96	39.60	254.29	KF735373		
GAV0849	UWBM 56350	*Eremophila alpestris*	*alpestris*	F	22-Jun-96	39.79	254.23	KF735402		
CSW6291	UWBM 72563	*Eremophila alpestris*	*alpestris*	M	9-Jun-01	40.58	255.27	KF735359		KF735539, KF735540
CSW6292	UWBM 72564	*Eremophila alpestris*	*alpestris*	M	6-Jul-00	40.58	255.27	KF735360		
CSW6293	UWBM 72565	*Eremophila alpestris*	*alpestris*	M	22-Jun-00	40.58	255.27	KF735361		
EVL371	UWBM 72567	*Eremophila alpestris*	*alpestris*	M	20-May-00	40.58	255.27	KF735390		
EVL372	UWBM 72568	*Eremophila alpestris*	*alpestris*	M	5-Jun-01	40.58	255.27	KF735391		
EVL373	UWBM 72569	*Eremophila alpestris*	*alpestris*		9-Jul-01	40.70	255.22	KF735392		
EVL388	UWBM 72570	*Eremophila alpestris*	*alpestris*	M	18-May-01	40.58	255.27	KF735393		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
EVL389	UWBM 72571	*Eremophila alpestris*	*alpestris*	F	24-Jun-00	40.58	255.27	KF735394		
GKD200	UWBM 72572	*Eremophila alpestris*	*alpestris*	F	5-Jun-00	40.58	255.27	KF735404	KF735304	KF735563, KF735564
GKD201	UWBM 72573	*Eremophila alpestris*	*alpestris*	F	27-May-01	40.58	255.27	KF735405	KF735305	KF735565, KF735566
MLD042	UWBM 72574	*Eremophila alpestris*	*alpestris*	M	24-Jun-00	40.58	255.27	KF735440		
MLD043	UWBM 72575	*Eremophila alpestris*	*alpestris*	M	20-May-00	40.58	255.27	KF735441		
EVL766	UWBM 80629	*Eremophila alpestris*	*alpestris*	M	7-Jun-04	46.85	256.03	KF735396		
EVL769	UWBM 80632	*Eremophila alpestris*	*alpestris*	M	7-Jun-04	46.85	256.03	KF735397		
DHB2279	MBM 5161	*Eremophila alpestris*	*alpestris*	F	20-Jun-97	38.21	256.44	KF735374		
DHB2281	MBM 5155	*Eremophila alpestris*	*alpestris*	F	20-Jun-97	38.21	256.44	KF735375		
DHB2283	MBM 5154	*Eremophila alpestris*	*alpestris*	F	20-Jun-97	38.21	256.44	KF735376		
T208	UMMZ	*Eremophila alpestris*	*alpestris*		27-Jun-87	36.75	257.48	KF735490		
T1777	UMIMNH	*Eremophila alpestris*	*alpestris*		16-May-93	41.63	257.68	KF735489		
B2273	KUNHM	*Eremophila alpestris*	*alpestris*		28-Jun-00	38.17	263.59	KF735348		
JDW0097	BMUM 42513	*Eremophila alpestris*	*alpestris*	M	7-Mar-95	45.00	264.09	KF735430		
b3558	KUNHM	*Eremophila alpestris*	*alpestris*		15-Jun-00	39.98	264.81	KF735351		
b3548	KUNHM	*Eremophila alpestris*	*alpestris*		22-May-00	38.10	265.64	KF735350		
SVD2851	BMUM	*Eremophila alpestris*	*alpestris*		18-Oct-02	47.50	266.42	KF735477		
SVD2852	BMUM	*Eremophila alpestris*	*alpestris*		18-Oct-02	47.50	266.42	KF735478		
SVD2854	BMUM	*Eremophila alpestris*	*alpestris*			45.03	267.08	KF735479		
T1775	UMMZ	*Eremophila alpestris*	*alpestris*		11-Dec-93	45.56	275.32	KF735488		
HOLA12	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735406		KF735567, KF735568
HOLA15	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735407		
HOLA17	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735408		
HOLA19	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735409		
HOLA22	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735410		
HOLA26	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735411		KF735569, KF735570
HOLA32	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735412		
HOLA33	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735413		
HOLA40	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735414		
HOLA52	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735415		
HOLA72	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735416		
HOLA78	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735417		
HOLA97	UGA	*Eremophila alpestris*	*alpestris*		egg shell	33.96	276.62	KF735418		
1B446	ROM	*Eremophila alpestris*	*alpestris*			43.65	280.62	KF735335		
1B592	ROM	*Eremophila alpestris*	*alpestris*			43.65	280.62	KF735336		
1B593	ROM	*Eremophila alpestris*	*alpestris*			43.65	280.62	KF735337		

Appendix S1. Samples used in this study and GenBank accession numbers

ID	Institution	Species	mtDNA clade	Sex	Date	Lat.	Lon.	ND2	ACO1	RHO
1B769	ROM	*Eremophila alpestris*	*alpestris*			43.65	280.62	KF735338		
1B771	ROM	*Eremophila alpestris*	*alpestris*	M		43.65	280.62	KF735339	KF735306, KF735307	KF735515, KF735516
SVD2384	UWBM 68259	*Eremophila alpestris*	*alpestris*	M	15-Mar-92	42.67	283.46	KF735476	KF735308, KF735309	KF735609, KF735610
SVD2361	UWBM 68254	*Eremophila alpestris*	*alpestris*	F	16-Aug-00	46.66	306.93	KF735473	KF735310	KF735605, KF735606
SVD2362	UWBM 68255	*Eremophila alpestris*	*alpestris*	F	16-Aug-00	46.66	306.93	KF735474		KF735607, KF735608
SVD2363	UWBM 68253	*Eremophila alpestris*	*alpestris*		16-Aug-00	46.66	306.93	KF735475		