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# The published complete mitochondrial genome of Blue-fronted Redstart (*Phoenicurus frontalis*) is a chimera and includes DNA from Pink-rumped Rosefinch *Carpodacus waltoni eos* (Aves: Passeriformes)

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## ABSTRACT

The complete mitochondrial genome of Blue-fronted Redstart (*Phoenicurus frontalis*), GenBank accession number MT360379 (NC\_053917), was published by Li and colleagues in 2020. Here we show that this mitogenome is actually a chimera containing DNA fragments of both *P. frontalis* (15,518 bp, 92.5%) and Pink-rumped Rosefinch (*Carpodacus waltoni eos*, 1258 bp, 7.5%). This mitogenome has been re-used in at least three phylogenies. Our study confirms that mitogenomes are best verified with multiple gene trees, and that any anomalies should be investigated by direct comparison of sequences.

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Chimerism; laboratory errors; mitogenome; sequence artifacts; Muscicapidae

## Introduction

Blue-fronted Redstart *Phoenicurus frontalis* (Vigors 1831) is a member of the mostly Old World bird family Muscicapidae, breeding in the Himalayas from north east Afghanistan east to Guizhou (China), and wintering at lower levels in northern India east to northern Indochina (Collar 2020). The first published mitochondrial genome sequence (hereafter mitogenome) of this species was published by Li et al. (2020). This sequence was derived from a sample obtained in Chaqing Songduo Nature Reserve, Baiyu County, Sichuan Province, China (GenBank accession number MT360379/reference sequence NC\_053917). Li et al. (2020) included a phylogram based on sequences of 12 protein-coding genes which placed the *P. frontalis* sequence sister to *P. aureus* with 1.0 posterior probability. Using a strategy described by Sangster & Luksenburg (2020), we show that accession MT360379 is actually a chimera containing DNA of two species of oscine passerines (*Phoenicurus frontalis* and *Carpodacus waltoni eos*).

## Materials and methods

We verified the identity of MT360379 by performing separate phylogenetic analyses of three protein-coding genes and comparing the position of each species in the gene trees: NADH dehydrogenase subunit 2 (*ND2*, 1041 bp), cytochrome oxidase subunit 1 (*CO1*, 1551 bp), and cytochrome b (*CYB*, 1143 bp). MITOS2 (Bernt et al. 2013), as implemented on the Usegalaxy website (The Galaxy Community 2022), was used to obtain information on the first and last positions of

individual genes. MUSCLE (as implemented in MEGA7, Kumar et al. 2016) was used to align sequences.

When we noticed that one of these data sets (*ND2*) showed a different, but strongly supported, position of *Phoenicurus frontalis* than the other data sets, we visually compared the complete *Phoenicurus frontalis* sequence with those of two other *Phoenicurus* sequences (KF997863, Zhang et al. 2018; MN122900, unpublished) and the mitogenome of *Carpodacus pulcherrimus* (MT648821, Yang et al. 2020) and determined the first and last positions of the anomalous fragment (Sangster and Luksenburg 2021a). We constructed separate phylogenies of (i) the anomalous fragment (1258 bp) and (ii) the rest of the mitogenome (15,518 bp). Maximum Likelihood phylogenies were obtained using IQ-tree version 2.2.2.6 (Minh et al. 2020). We partitioned the data sets in multiple categories: ribosomal RNA, transfer RNA, protein-coding genes (each partitioned by codon) and control region. The appropriate substitution model for each partition (or appropriate combinations of partitions) in the two data sets was selected using ModelFinder (Kalyaanamoorthy et al. 2017). Branch support was obtained using ultra-fast bootstrapping (Hoang et al. 2018). Sequence divergence was calculated as uncorrected p-value with complete deletion of nucleotide positions with missing data.

The data set included the following sequences: *Carpodacus erythrinus* KM078766 (Lerner et al. 2011), *Carpodacus pulcherrimus* MT648821 (Yang et al. 2020), *Carpodacus roseus* KM078779 (Lerner et al. 2011), *Carpodacus rubicilloides* MH363715 (Qin et al. 2019), *Chloris sinica* MH047558 (Kim et al. 2018),

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*Copsychus saularis* KU058637 (Peng et al. 2016), *Fringilla coelebs* KM078769 (Marshall et al. 2013), *Menura novaehollandiae* AY542313 (Slack et al. 2007), *Oenanthe oenanthe* MT431000 (Wang et al. 2020), *Phoenicurus aureus* KF997863 (Zhang et al. 2018), *Phoenicurus frontalis* MT360379 (Li et al. 2020), *Phoenicurus phoenicurus* MN122900 (unpublished) and *Turdus obscurus* MZ337397 (Zhang et al. 2021). We used *Cnemotriccus fuscatus* AY596278 (Slack et al. 2007) and *Mionectes oleagineus* KJ742591 (Loaiza et al. 2016) as outgroups.

## Results

Initial analysis, based on gene trees of *ND2*, *CO1*, and *CYB*, showed that in the *ND2* gene tree, *Phoenicurus frontalis* (MT360379) clustered with Himalayan Beautiful Rosefinch *Carpodacus pulcherrimus* (JN715431, Zuccon et al. 2012; KF194131, KF194141, KF194154, KF194155, KF194156, KF194159, Tietze et al. 2013; MT648821, Yang et al. 2020), with strong support, whereas the other two gene trees placed *Phoenicurus frontalis* (MT360379) with reference sequences of *P. frontalis* (*CO1*: JX970703, Hogner et al. 2012; *CYB*: AY206917, Beresford 2003; DQ285428, Pan et al. 2006; GU930801, Voelker 2010; HM633363, Sangster et al. 2010; KJ456387, Price et al. 2014).

Direct (visual) comparison of the mitogenome sequences showed that the anomalous part consisted of a single 1258 bp fragment, located at positions 3797–5054. This represented 7.5% of the total length of MT360379 (16,776 bp) and included *ND2*, *trnQ(ttg)*, *trnM(cat)*, and parts of *trnI(gat)* and *trnW(tca)*. A Maximum Likelihood (ML) phylogeny of this portion of the mitogenome is shown in Figure 1(a), which shows a sister-relationship of mitogenome MT360379 and *C. pulcherrimus* with 100% bootstrap support. Sequence divergence between this portion of the mitogenomes of MT360379 and *C. pulcherrimus* was small (0.1%). A ML phylogeny of the other parts of the mitogenome is shown in Figure 1(b), which

placed MT360379 with sequences of two other species of *Phoenicurus* with 100% bootstrap support.

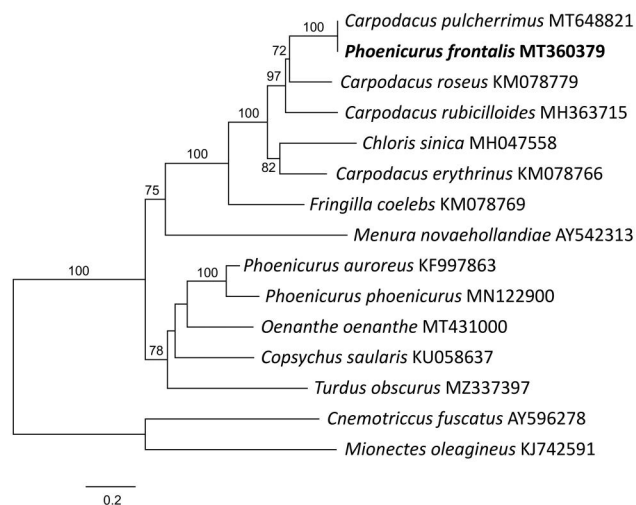
## Discussion

Our results show that different parts of MT360379 cluster with different species, each with strong bootstrap support. A large portion of mitogenome MT360379 (i.e. positions 1–3796 and 5055–16,776) was closely related to other mitogenomes of *Phoenicurus* (Muscicapidae), of which the *CO1* and *CYB* fragments clustered with reference sequences of *P. frontalis*. However, another fragment (positions 3797–5054) was clearly that of *C. pulcherrimus*, a member of a distantly related clade of passerines (Fringillidae). MT360379 thus represents a chimera of *P. frontalis* and *C. pulcherrimus* (but see below).

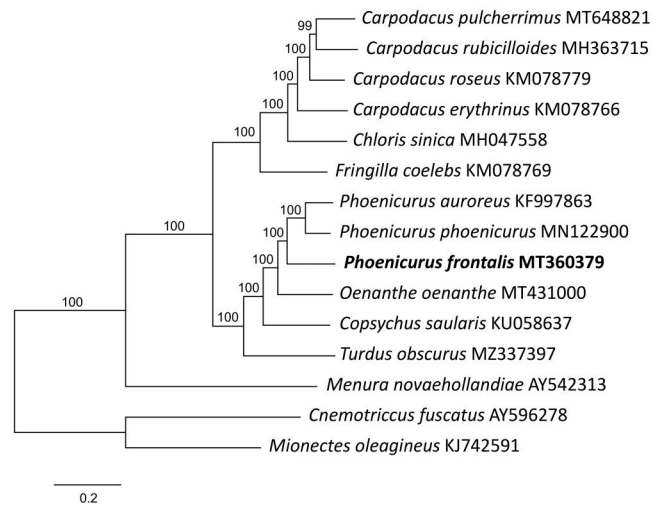
The taxonomy of *C. pulcherrimus* was recently revised, which led to the recognition of three species, *C. pulcherrimus*, *C. davidianus* and *C. waltoni* (Dickinson and Christidis 2014; Gill et al. 2024). Following this revision, the mitogenome sequence originally published as '*C. pulcherrimus*' (MT648821) by Yang et al. (2020) represents *C. waltoni eos* because (i) it clustered most closely with *CYB* sequences of that taxon (data not shown), and (ii) because the locality where MT648821 was sampled (Baiyu County, Sichuan; Yang et al. 2020) is within the range of *C. waltoni eos* (Tietze et al. 2013). The very close relationship of positions 3797–5054 of the published mitogenome of *Phoenicurus frontalis* (MT360379) to '*C. pulcherrimus*' (MT648821) shows that this fragment also represents *C. waltoni eos*.

The published mitogenome of *Phoenicurus frontalis* (MT360379) was obtained with Sanger sequencing. The chimera likely occurred in the laboratory resulting from the transfer of a sample of *C. pulcherrimus* (= *C. waltoni eos*) to a tube intended for *P. frontalis* before PCR amplification or before DNA sequencing. Indeed, a mitogenome of *C. pulcherrimus* (= *C. waltoni eos*) was sequenced by members of the same team (Yang et al. 2020). Detecting such errors is

(a) bp 3797–5054



(b) bp 1–3796 and bp 5055–16,776



**Figure 1.** ML phylogenies of oscine passerines (passeriformes) based on (a) positions 3797–5054 (1258 bp) of mitogenome MT360379, (b) mitogenomes excluding positions 3797–5054. Numbers along branches represent bootstrap support values (>70%) based on 1000 pseudoreplications. Note the different position of *phoenicurus frontalis* (MT360379) in the two gene trees.

possible if multiple fragments are analyzed separately using phylogenetic methods (i.e. compared with reference sequences) before assembling the fragments into a single mitogenome.

We report this problematic mitogenome because accumulation of erroneous sequences may compromise subsequent applications, including DNA identification, primer design for intraspecific studies, phylogenetic inference, historical biogeography, taxonomy and comparative analysis (Sangster and Luksenburg 2021a). We found three re-uses of the mitogenome (Zhao et al. 2022, 2023; Lan et al. 2024). In all cases, the mitogenome was included in a phylogeny.

Our study, and that of Sangster and Luksenburg (2021a), underscore that chimeras are easily overlooked without dedicated analysis. We suspect that the few cases of chimerism reported so far in vertebrate mitogenomics (e.g. Norén and Kullander 2018; Sangster and Luksenburg 2020, 2021a, 2021b, 2023, 2024) do not reflect the true prevalence of this problem. It is clear that greater vigilance is necessary during laboratory procedures, quality control of raw data, and peer review of the final sequences.

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## Author contributions

George Sangster: conceptualization, methodology, formal analysis, investigation, and writing—original draft. Jolanda Luksenburg: writing—review and editing. Both authors agreed to be accountable for all aspects of the work and approved the final draft to be published.

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