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ORIGINAL ARTICLE

A Re-Assessment of Positive Selection on Mitochondrial Genomes of High-Elevation *Phrynocephalus* Lizards

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Abstract

Due to their integral roles in oxidative phosphorylation, mitochondrially encoded proteins represent common targets of selection in response to altitudinal hypoxia across high-altitude taxa. While previous studies revealed evidence of positive selection on mitochondrial genomes of high-altitude *Phrynocephalus* lizards, their conclusions were restricted by out-of-date phylogenies and limited taxonomic sampling. Using topologies derived from both nuclear and mitochondrial DNA phylogenies, we re-assessed the evidence of positive selection on the mitochondrial genomes of high-altitude *Phrynocephalus*. We sampled representative species from all four main lineages and sequenced the mitochondrial genome of *P. maculatus*, a putative sister taxon to the high-altitude group. Positive selection was assessed through two widely used branch-site tests: the branch-site model in PAML and BUSTED in HyPhy. No evidence of positive selection on mitochondrial genes was detected on branches leading to two most recent common ancestors of high-altitude species; however, we recovered evidence of positive selection on COX1 on the *P. forsythii* branch, which represents a reversal from high- to low-elevation environments. A positively selected site therein marked a threonine to valine substitution at position 419. We suggest this bout of selection occurred as the ancestors of *P. forsythii* re-colonized lower altitude environments north of the Tibetan Plateau. Despite their role in oxidative phosphorylation, we posit that mitochondrial genes are unlikely to have represented historical targets of selection for high-altitude adaptation in *Phrynocephalus*. Consequently, future studies should address the roles of nuclear genes and differential gene expression.

Keywords *Phrynocephalus* · Altitude · Elevation · Mitochondrial genome · Positive selection · Branch-site model

Abbreviations

OXPHOS	Oxidative phosphorylation
nuDNA	Nuclear DNA
mtDNA	Mitochondrial DNA
MRCA	Most recent common ancestor
bp	Base pairs
dN	Nonsynonymous substitution
dS	Synonymous substitution
LRT	Likelihood ratio test
BEB	Bayes Empirical Bayes
P_b	Posterior probability
PROVEAN	Protein Variation Effect Analyzer
ND	NADH Dehydrogenase

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Background

High-elevation adaptation represents a classic example of adaptive evolution, as high-altitude environments present significant challenges to organisms therein, including cold temperatures, high levels of ultraviolet radiation, and hypoxia (Cheviron and Brumfield 2012). While stressors exasperated by the two former conditions may be relieved through behavioural adaptations (Sinsch 1989; Bauwens et al. 1996; Ferguson et al. 2014), challenges associated with chronic hypoxia are frequently mitigated through genetic and physiological adaptations (Storz and Moryiama 2008; Cheviron and Brumfield 2012). While atmospheric oxygen concentration is ~21% at sea level, it drops to ~13% at altitudes of 4000 m. Mitochondrial proteins are associated with

oxidative phosphorylation (OXPHOS), ultimately generating the vast majority of adenosine triphosphate through the electron transport chain, wherein the diatomic oxygen molecule represents the final electron acceptor (Soltoff 1986). Accordingly, previous studies have revealed evidence of positive selection on mitochondrial genes, in response to altitudinal hypoxia (Hassanin et al. 2009; Scott et al. 2011; Yu et al. 2011).

Toad-headed lizards of the genus *Phrynocephalus* represent an excellent study system for high-elevation adaptation. Distributed across central Asia to the Middle East, the genus *Phrynocephalus* comprises over 30 species; four of them (*P. erythrurus*, *P. putjatai*, *P. theobaldi*, and *P. vlangalii*) are primarily distributed across the Tibetan Plateau region. Several of these species have physiological adaptations enabling them to withstand the environmental extremes associated with their high-elevation habitats (Tang et al. 2013). Two recent studies assessed the genetic basis of high-altitude adaptation in *Phrynocephalus* by examining positive selection on mitochondrial genes (Li et al. 2015; Jin et al. 2018). Both used PAML (Yang 2007), a phylogeny-based analysis, and both indicated that positive selection on mitochondrially encoded proteins may have played a crucial role in facilitating high-altitude adaptation in *Phrynocephalus* (Li et al. 2015; Jin et al. 2018). For example, evidence strongly suggested that positive selection occurred at ten codon sites (Jin et al. 2018). Nevertheless, both studies suffered from two limitations: an out-of-date phylogeny and limited sampling.

Through a highly supported nuclear DNA (nuDNA) phylogeny, Solovyeva et al. (2018) revealed novel insights into the *Phrynocephalus* phylogeny, most notably the presence of four major clades. All the Tibetan species formed a clade; this is consistent with all previous phylogenetic studies (Guo and Wang 2007; Jin and Brown 2013; Solovyeva et al. 2014). All five Tibetan species are viviparous, and all are distributed at high elevations, except *P. forsythii* (Chen et al. 2016; Jin et al. 2018). All species distributed in west Asia (i.e. Arabian Peninsula, Iranian Plateau) formed the second clade. *P. mystaceus* solely comprised the third clade, while the remaining central Asian species formed the fourth (Fig. 1a). While the constituent species of the major groups are well-supported, the relationships among them are not. Furthermore, the nuDNA phylogeny contrasted mitochondrial DNA (mtDNA) phylogenies (Fig. 1b) (Guo and Wang 2007; Jin and Brown 2013; Solovyeva et al. 2014; Macey et al. 2018; Solovyeva et al. 2018). Several documented biological phenomena, including mitochondrial genome introgression (Noble et al. 2010; Solovyeva et al. 2018) and sex-biased dispersal (Urquhart et al. 2009), likely caused the deviation of the mtDNA tree from the nuDNA species tree. Both early studies were based on mtDNA-derived phylogenies (Fig. 1c) and treated the Tibetan clade as the sister group to the remainder of the genus (Li et al. 2015; Jin et al. 2018). Furthermore, both studies sampled only three of the four major lineages (Li et al. 2015; Jin et al. 2018); considering the uncertainly of the relationships among the four major lineages, the omission of the west Asian group in previous studies could be significant.

We re-assessed the presence of positive selection on the mitochondrial genes of high-altitude *Phrynocephalus* with three improvements. Firstly, we used the new nuDNA-based phylogeny of Solovyeva et al. (2018). Considering the uncertainty among the four major groups, we also analyzed the data with an additional mtDNA topology corresponding

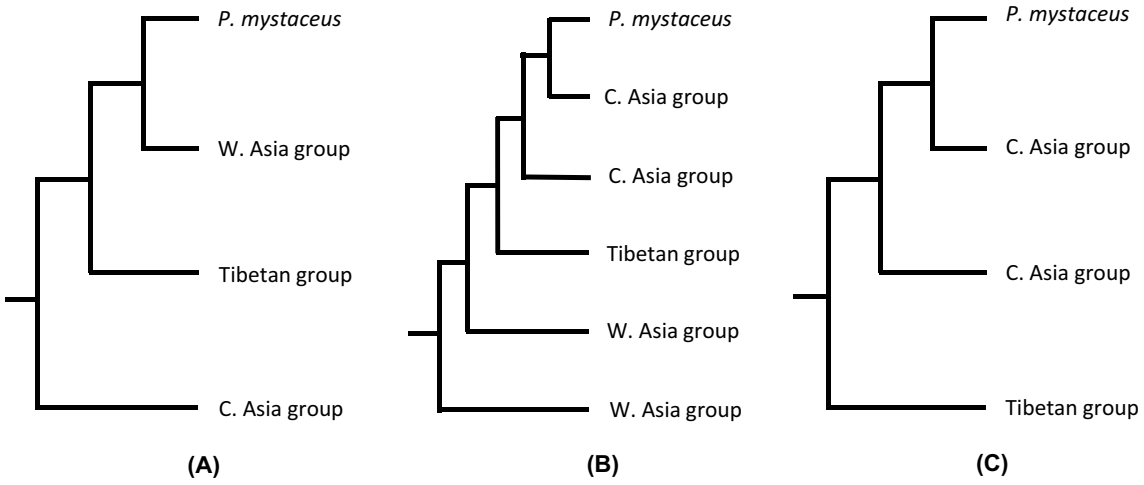


Fig. 1 Alternative tree topologies (simplified) of the genus *Phrynocephalus*. The designations "C. Asia" and "W. Asia" represent central and west Asia, respectively. **a** Tree derived from four nuclear genes: RAG-1, BDNF, AKAP9, and NKTR (Solovyeva et al. 2018). **b** Tree derived from four mitochondrial genes: COX1, CYTB, ND2, and ND4 (Solovyeva et al. 2018). **c** Tree used in previous analyses of positive selection on mitochondrial genomes of *Phrynocephalus*, derived from mtDNA (Li et al. 2015; Jin et al. 2018)

to that recovered by Solovyeva et al. (2018). Secondly, we added a sample representing the west Asian group, which was absent from the previous studies (Li et al. 2015; Jin et al. 2018). Thirdly, we focused our analyses on three branches: the most recent common ancestor (MRCA) of the Tibetan group; the MRCA of *P. erythrurus*, *P. forsythii*, and *P. theobaldi*; and *P. forsythii* itself (Fig. 2a). Since all Tibetan species form a clade nested within low elevation lineages, the adaptive changes, if any, would be expected to occur along the branch leading to the MRCA of the Tibetan species. Furthermore, two species, *P. erythrurus* and *P. theobaldi*, are distributed across elevations considerably higher than the remainder of the Tibetan clade, and previous studies recovered evidence of positive selection along the *P. theobaldi* lineage (Li et al. 2015; Jin et al. 2018). Therefore, adaptive changes would also be expected along the branch corresponding to the MRCA of *P. erythrurus*, *P. forsythii*, *and P. theobaldi*. Of the Tibetan group, *P. forsythii* is the only low elevation species (Jin et al. 2018); since it is nested within the high-altitude clade, it most likely evolved from a high-elevation ancestor. Therefore, it provides an opportunity to evaluate a reversal, which often provides strong evidence for the initial evolutionary change. By defining the focal branches *a priori*, our hypothetico-deductive approach should be more powerful to address the underlying questions.

Methods

DNA Extraction, Amplification, and Sequencing

We sequenced the 13 protein-coding genes from the mitochondrial genome of *P. maculatus*, a representative from the west Asian lineage (Solovyeva et al. 2018). DNA was extracted using the standard phenol–chloroform protocol (Sambrook et al. 1989). DNA was amplified by PCR using primers available from Li et al. (2015), as well as primers designed in this study (Supplementary Table S1). An annealing temperature of 50 °C was used for all PCR reactions. Amplified PCR products were purified using the AxyPrep PCR clean-up protocol and were sequenced on an ABI 3730 automated sequencer using both forward and reverse primers.

Data Collection and Analysis

Complete mitochondrial genomes of nine *Phrynocephalus* species, and two outgroup species, *Pseudotrapelus sinaitus* and *Xenagama taylori*, were downloaded from GenBank. Together with *P. maculatus*, these ten species represent all four major lineages within the genus (Fig. 2; Supplementary Table S2). All 13 protein-coding genes were individually aligned in separate NEXUS files, and all alignments were completed using MUSCLE with manual adjustments to account for frame-shifting gaps and/or sequencing errors (Edgar 2004).

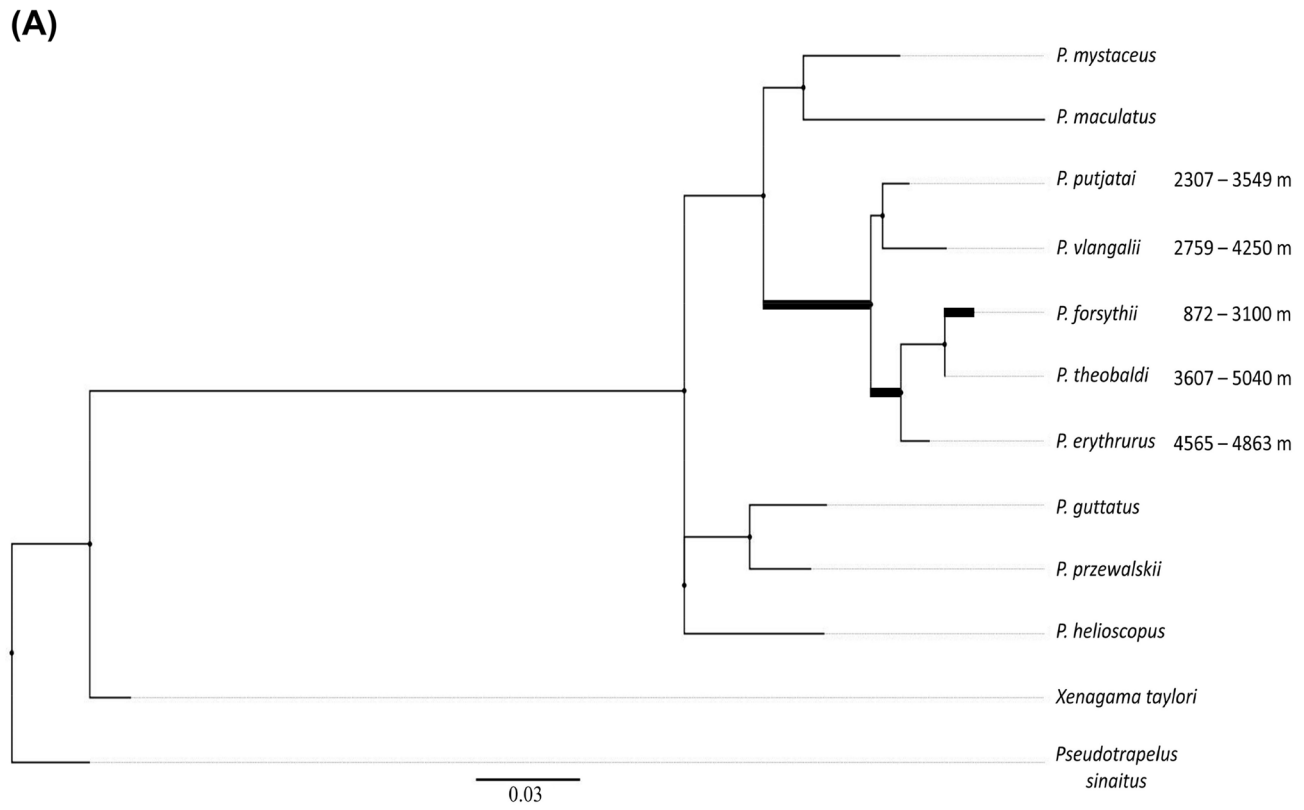
We primarily used the phylogenies of the genus *Phrynocephalus* from Solovyeva et al. (2018). Due to the uncertainty of relationships among the four major lineages, we constructed two topologies to incorporate different lineages as the sister group to the high-altitude clade for downstream analysis (Fig. 2). Topology 1 is fully compatible with the nuDNA topology of Solovyeva et al. (2018), featuring *P. maculatus* and *P. mystaceus* as sister taxa to the high-altitude clade. Topology 2 followed the mtDNA phylogeny recovered by Solovyeva et al. (2018) and placed the clade comprised *P. mystaceus*, *P. guttatus*, *P. helioscopus*, and *P. przewalskii* as the sister group to the high-altitude clade. These two topologies represent the most likely phylogenies of the ten species based on previous studies (Guo and Wang 2007; Jin and Brown 2013; Solovyeva et al. 2014; Macey et al. 2018; Solovyeva et al. 2018).

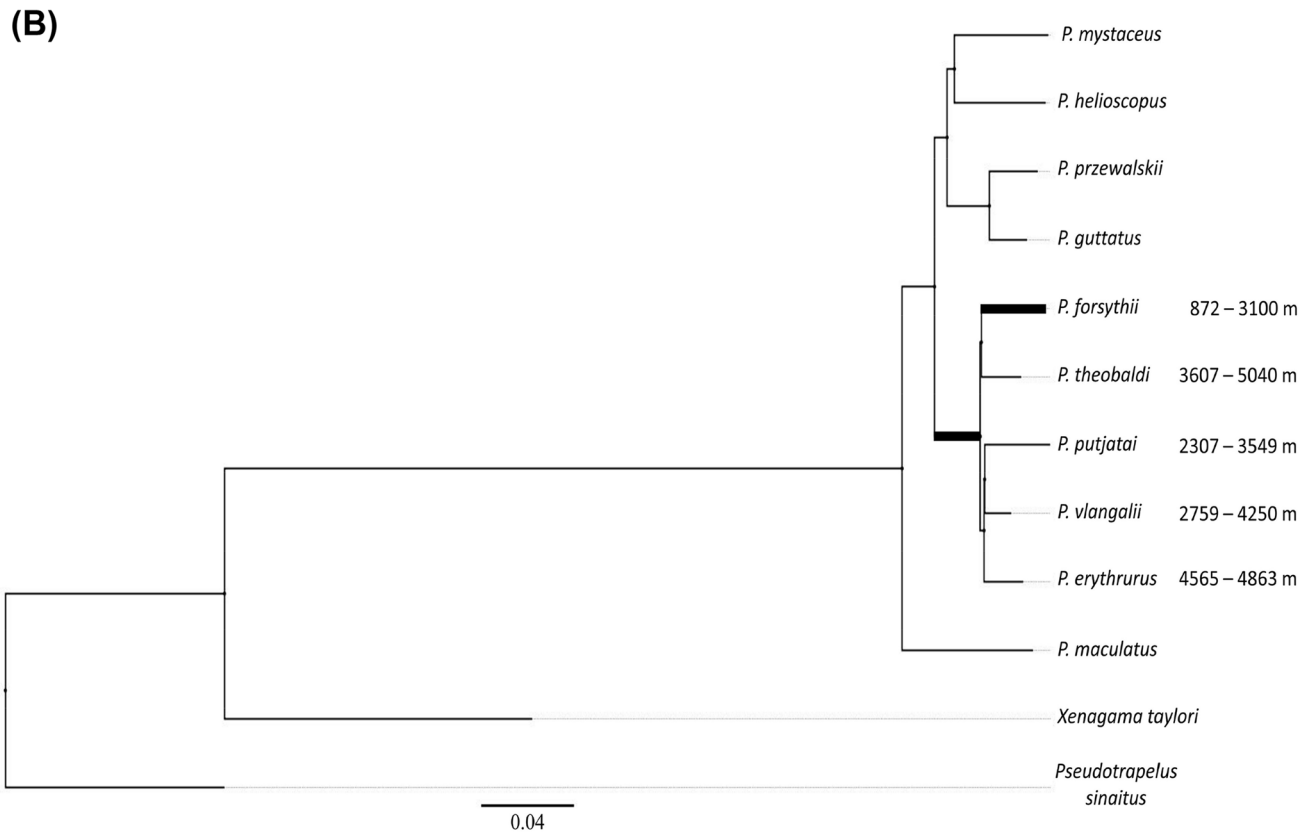
We used DNA sequences of four nuclear genes, RAG-1, BDNF, AKAP9, and NKTR, to estimate the branch lengths for the nuDNA topology (Topology 1; Supplementary Table S3). The same sequence data were used to estimate the nuDNA phylogeny by Solovyeva et al. (2018). For the mtDNA phylogeny (Topology 2), we used the concatenated alignment of the 13 protein-coding mitochondrial genes (11284 base pairs (bp)) to estimate branch lengths. Data were obtained from GenBank, and sequences were aligned and concatenated in Mesquite v3.31 (Maddison and Maddison 2017). The TIM + I + G substitution model was selected for the nuDNA topology and the GTR + I + G substitution model was selected for the mtDNA topology. All branch lengths were estimated using PAUP* 4.0a164 (Swofford 2003).

The CODEML analysis (in PAML package) requires an unrooted tree, and therefore we collapsed the node joining the outgroups. Additionally, all branch lengths were multiplied by three for codon-based analyses, as suggested by Yang (2005).

Tests of Positive Selection

Gene-wide positive selection was assessed using two methods: the branch-site model in CODEML (Yang and Nielsen 2002; Zhang et al. 2005), implemented in PAML (Yang 2007) and the branch-site unrestricted test for episodic diversification (BUSTED) (Murrell et al. 2015), implemented in HyPhy (Kosakovsky Pond et al. 2005). Both methods detect the presence of gene-wide positive selection by assessing the ratio of nonsynonymous (dN) to synonymous substitutions (dS) on *a priori*-defined focal, or foreground, branches (Yang and dos Reis 2011; Murrell et al. 2015). The frameworks underlying


(A)
P. mystaceus
P. maculatus
P. putjatai 2307 – 3549 m
P. vlangalii 2759 – 4250 m
P. forsythii 872 – 3100 m
P. theobaldi 3607 – 5040 m
P. erythrurus 4565 – 4863 m
P. guttatus
P. przewalskii
P. helioscopus
Xenagama taylori
Pseudotrapelus sinaitus
0.03


(B)
P. mystaceus
P. helioscopus
P. przewalskii
P. guttatus
P. forsythii 872 – 3100 m
P. theobaldi 3607 – 5040 m
P. putjatai 2307 – 3549 m
P. vlangalii 2759 – 4250 m
P. erythrurus 4565 – 4863 m
P. maculatus
Xenagama taylori
Pseudotrapelus sinaitus
0.04

◂**Fig. 2** Phylogenies used in selection analyses of *Phrynocephalus* mitochondrial genes. Bold branches represent focal (foreground) branches. Altitudinal ranges of Tibetan species listed to the right of the taxon name (Jin et al. 2018). **a** The nuDNA-derived topology. **b** The mtDNA-derived topology

each method differ considerably, and they often recover different results on a single dataset (Nozawa et al. 2009). The branch-site model in CODEML compares the likelihood under positive selection with the likelihood under the null hypothesis, using a likelihood ratio test (LRT). Furthermore, the site-specific Bayes Empirical Bayes (BEB) method was used to identify individual codon sites putatively under positive selection and assign them corresponding posterior probability (*Pb*) values (Yang et al. 2005; Zhang et al. 2005). This method calculates the posterior probability of sites with dN/dS > 1 (Yang et al. 2005). BUSTED assesses selection on a subset of sites along the gene of interest and incorporates stochastic variation among branch classes (Murrell et al. 2015). As such, it possesses enhanced statistical power to assess selection events that could be transient or localized to certain sites within the gene (Murrell et al. 2012). Ultimately, BUSTED aims to test if at least one site on any foreground branch has undergone positive selection (Murrell et al. 2015). Each method was conducted three times, to detect putative positive selection on each foreground branch: the branch corresponding to the MRCA of the high-altitude clade; the branch leading to the MRCA of *P. erythrurus*, *P. forsythii*, and *P. theobaldi*; and the branch leading to *P. forsythii*.

Codon sites with $Pb \geq 0.95$ recovered by the BEB method were further assessed using the Protein Variation Effect Analyzer (PROVEAN) webserver (Choi and Chan 2015). Herein, the ancestral amino acid sequence generated in CODEML and the amino acid substitution(s) specified by the BEB method were used to estimate the impact of the substitutions on the protein's function. PROVEAN clusters the input amino acid sequence with homologs from the NCBI non-redundant protein database (September 2012) (Pruitt et al. 2012) through the basic local alignment search tool, forming a supporting sequence set of the top 30 clusters of highly similar sequences to produce a prediction regarding the effect of the given mutation(s) (Choi et al. 2012). The analysis computes a PROVEAN score with a threshold of − 2.5, wherein the mutation is predicted to be deleterious if the score ≤ − 2.5, while a score > − 2.5 indicates that the variant is predicted to have a neutral effect on the protein's function.

Results

All 13 protein-coding mitochondrial genes of *P. maculatus* were sequenced. Gene lengths ranged from 159 base pairs in ATP8 to 1767 bp in ND5 and were highly concordant with sequences obtained from its congeners (Supplementary Table S2).

Tests of positive selection on each gene revealed one instance of a significantly elevated gene-wide dN/dS ratio, through the branch-site model in CODEML. We detected significant evidence of positive selection on COX1 along the branch leading to *P. forsythii* using the mtDNA topology (LRT = 4.13; $p = 0.042$). Similarly, the evidence was nearly significant when the nuDNA topology was used (LRT = 3.80; $p = 0.051$) (Supplementary Table S4). Significant evidence of positive selection was not revealed through BUSTED.

The BEB method detected evidence of several putative positively selected sites with significant posterior probabilities (Table 1). However, unless the LRTs from the corresponding branch-site tests reach statistical significance, these site-specific results cannot be interpreted as evidence for positive selection, regardless of their high posterior probabilities (Zhang et al. 2005). A threonine to valine substitution at codon position 419 of COX1 was observed on the *P. forsythii* branch using the nuDNA topology (Table 1). PROVEAN predicted that most substitutions were of neutral effect, and only two substitutions were likely involved in functional change (deleterious). The substitution on codon position 419 of COX1 was predicted to be neutral (Table 1).

Discussion

Overall, the branch-site model revealed a signal of positive selection on the COX1 gene of the mitochondrial genome of *P. forsythii*, following divergence from its MRCA with *P. theobaldi*. A threonine to valine substitution at codon site 419 of COX1 is likely the result of positive selection (BEB $Pb = 0.993$; branch-site model $p = 0.051$). We did not detect any clear signal of positive selection on branches leading to high-elevation species, as previously reported (Li et al. 2015; Jin et al. 2018).

It is difficult to predict the fitness benefit of the substitution within the COX1 gene of *P. forsythii*. The COX genes have been implicated in high-elevation adaption in the bar-headed goose (*Anser indicus*), wherein a single tryptophan to arginine substitution within COX3 was reported to alter its interaction with COX1 (Scott et al. 2011). However, considering that *P. forsythii* is primarily distributed at elevations below 2000 m (Chen et al. 2016), it is unlikely that the detected bout of selection conferred adaptation to the hypoxic extremes of the Tibetan Plateau. Evolutionary trade-offs between metabolic rate and adaptation to cold and hypoxic conditions are common in ectothermic taxa, including *Phrynocephalus* (Tang et al. 2013; Geisler et al. 2017). For instance, the mitochondrial respiratory rate of *P. erythrurus* is significantly lower than that of a lowland

Table 1 Putative positively selected codon sites with $P_b \geq 0.95$, recovered by the BEB method

Input topology	Gene	Focal branch	Position	Ancestral amino acid	Novel amino acid	Posterior probability (P_b)	PROVEAN score	PROVEAN prediction
mtDNA	ATP6	MRCA	50	A	M	0.952	1.852	Neutral
nuDNA	ATP6	*Forsythii*	11	T	I	0.991	−1.899	Neutral
nuDNA	ATP8	*Forsythii*	11	S	M	0.993	−2.500	Deleterious
nuDNA	**COX1**	***Forsythii***	**419**	**T**	**V**	**0.993**	**1.408**	**Neutral**
nuDNA	COX2	*Forsythii*	53	V	T	0.952	2.664	Neutral
nuDNA	COX2	*Forsythii*	91	A	N	0.952	4.278	Neutral
nuDNA	CYTB	*Forsythii*	30	M	A	0.962	0.875	Neutral
nuDNA	CYTB	*Forsythii*	47	I	V	0.956	−0.395	Neutral
nuDNA	CYTB	*Forsythii*	308	A	I	0.964	1.727	Neutral
nuDNA	ND2	*Forsythii*	14	I	A	0.979	−1.434	Neutral
nuDNA	ND2	*Forsythii*	155	L	T	0.976	−3.498	Deleterious
nuDNA	ND3	*Forsythii*	98	L	M	0.957	−1.311	Neutral
nuDNA	ND4	*Forsythii*	36	A	L	0.970	0.834	Neutral
nuDNA	ND5	*Forsythii*	44	L	P	0.966	6.883	Neutral
nuDNA	ND5	*Forsythii*	178	T	V	0.982	−1.017	Neutral
nuDNA	ND5	*Forsythii*	253	A	T	0.953	−0.282	Neutral
nuDNA	ND5	*Forsythii*	471	M	A	0.980	−0.523	Neutral
nuDNA	ND6	*Forsythii*	114	L	P	0.973	−2.278	Neutral

Mutations were deemed neutral if the PROVEAN score exceeded the cut-off of −2.5. Positively selected codon sites corresponding to an LRT approaching significance are bolded

congener, *P. przewalskii*, even with an increased incubation temperature (Tang et al. 2013). As a reduced metabolism may result in a competitive disadvantage to ectothermic species living in warmer, normoxic environments, we suggest that the positive selection observed on COX1 may have conferred adaptation enabling the high-altitude ancestors of *P. forsythii* to re-colonize the lower elevations of the Taklamakan Desert. Alternatively, the substitution may have no functional consequences and the detection may represent a false positive. PROVEAN predicted that the substitution likely has a neutral effect on the protein's overall function, although it exchanges a polar amino acid with a nonpolar amino acid.

With a new phylogeny and additional samples, our findings largely contrasted several previous studies. Li et al. (2015) detected signals of positive selection on the ATP8 gene in the high-elevation species *P. theobaldi*, and Jin et al. (2018) revealed strong evidence of positive selection on five genes (COX3, ND2, ND4, ND5, ND6) of *P. theobaldi*. Both subscribed the changes to high-elevation adaptation. We did not detect any signal of positive selection indicative of high-elevation adaptation.

The BEB method recovered numerous codon sites across different genes with significantly elevated site-specific dN/dS ratios (Table 1). Without a gene-wide dN/dS ratio > 1, they could be caused by relaxed purifying selection (Zhang et al. 2005; Wertheim et al. 2015). Furthermore, Jin et al. (2018) also detected positive selection on the ND4 gene of *P. forsythii*; instead, we detected significant positive selection on the COX1 gene of *P. forsythii*. Nevertheless, we did not detect any signals of positive selection along the MRCA branch leading to all high-elevation species, which is concordant with previous studies (Li et al. 2015; Jin et al. 2018).

The lack of signals of positive selection along the MRCA branch is not particularly surprising. Firstly, though a large body of research has implicated mitochondrially encoded proteins as targets of selection for altitudinal hypoxia across phyla, due to their integral role in OXPHOS (Scott et al. 2011; Zhou et al. 2014; Li et al. 2018; Shi et al. 2018; Yuan et al. 2018), it is notable that the nuclear genomes of most animals encode roughly 80 proteins involved in OXPHOS (Nicholls and Ferguson 2013). In fact, a recent study indicated that positive selection on several nuclear OXPHOS genes may have facilitated high-altitude adaptation in *Phrynocephalus* (Jin et al. 2020). Secondly, although dN/dS ratio is frequently used to assess selection (Yang et al. 2002; Zhang et al. 2005; Murrell et al. 2015), gene-wide dN/dS ratios exceeding 1 are infrequent (Hughes 2007). Instead, adaptive evolution is postulated to be driven by changes to single amino acids, gene deletions, altered gene expression, and gene duplications (Hughes 2007; Kondrashov 2012). As such, further studies may seek to analyze selection at the population-level, which has greater sensitivity to detect evolutionary change than dN/dS ratio-based tests

(Kryazhimskiy and Plotkin 2008; Lotterhos and Whitlock 2014). Additionally, gene expression level differences should be further explored (López-Maury et al. 2008; Yang et al. 2014); a recent comparative transcriptome analysis revealed three genes involved in hypoxic response in *P. vlangalii* (Yang et al. 2014).

A reliable phylogeny is fundamental to tree-based detection of positive selection. Our results, which vary considerably from previous analyses using different trees, clearly demonstrate its importance. Solovyeva et al. (2018), which used many species and both mitochondrial and nuclear genes, represents the best hypothesis thus far and is a major improvement from previous mtDNA-derived phylogenies. Nevertheless, disagreements remain, particularly regarding the relationships among the four main groups (Jin et al. 2020). Considering the well-documented limitations of mitochondrial genes in phylogenetic reconstructions of *Phrynocephalus* (Urquhart et al. 2009; Noble et al. 2010; Solovyeva et al. 2018), use of genomic data appears to be the logical next step to establish a reliable phylogeny for the genus.

Conclusions

The genetic bases of high-altitude adaptation represent a significant area of research to evolutionary biologists and physiologists alike. While behavioural mechanisms may be employed to cope with extreme climate and ultraviolet radiation (Sinsch 1989; Bauwens et al. 1996; Ferguson et al. 2014), overcoming altitudinal hypoxia requires genetic and physiological adaptations, with mitochondrially encoded proteins representing common targets of selection across taxa (Scott et al. 2011; Yuan et al. 2018). Although previous research indicated positive selection on mitochondrial genes of several lineages within *Phrynocephalus* (Li et al. 2015; Jin et al. 2018), the conclusions were limited by methodological constraints. Through the *a priori* designation of focal branches; incorporation of multiple topologies; and the inclusion of *P. maculatus*, a representative from a previously unsampled clade, our analysis found evidence of positive selection on COX1 on the *P. forsythii* branch. However, as no evidence of positive selection was observed on the branch corresponding to the MRCA of high-altitude *Phrynocephalus*, and the bout of positive selection on COX1 was associated with the transition from high-altitude to low-altitude habitats, we posit that selection on mitochondrial genes did not confer high-altitude adaptation in *Phrynocephalus*.

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Authors Contribution JEA contributed lab work, statistical analyses and drafted the manuscript. JF designed the study, contributed lab work, and edited the manuscript. Both authors read and approved the final manuscript.

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Data Availability All sequences generated in this study have been deposited in GenBank (Accession Number: MW007749).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval and Consent to Participate All animal utility protocols are approved by the Animal Care Committee of the University of Guelph (AUP#3886).

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