

## ORIGINAL ARTICLE

# Multilocus phylogeny reveals habitat driven cryptic diversity in *Ochotona rufescens* (Ochotonidae)

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## Abstract

Taxonomy of Afghan pikas *Ochotona rufescens* distributed in Western and Central Asia has long been debated, and the presence of cryptic diversity within the species has been suggested. In this study, we used one mitochondrial (cyt *b*) and six nuclear (nuDNA) markers to investigate genetic diversity within Afghan pikas *O. rufescens* populations based on 54 new samples from Western and Central Asia. In total, we included 200 new sequences of mitochondrial cyt *b* and six nuclear markers from 60 samples. Our results reveal a genetic split within Afghan pikas both in mitochondrial and nuclear markers, estimated to date back to at least 0.58 Mya, suggesting that two cryptic and isolated lineages exist among Afghan pikas in Western and Central Asia. Geographic patterns corresponding to known subspecies were largely corroborated. We hypothesize that during interglacials pikas are forced to track their habitat and move to higher elevations to avoid the desertification of the lowlands, effectively trapping them in refugia at the highest elevations. During glacials, cool climate may have created lowland habitats that pikas could tolerate, potentially allowing them to disperse between mountains. Pikas thus appear to be forced into refugia during the warmer climates of interglacials, rather than enduring ice ages in refugia, like most other organisms discussed in the context of Pleistocene refugia.

## KEYWORDS

Afghan pikas, interglacial refugia, Lagomorpha, mitochondrial DNA, nuclear DNA, Pleistocene, Western Asia

## 1 | INTRODUCTION

Pikas of the genus *Ochotona* Link, 1795 (Lagomorpha: Ochotonidae) are relatively small lagomorphs representing more than 32 species occupying the northern hemisphere, comprising five subgenera including *Ochotona*, *Pika*, *Conothoa*, *Lagotona* and *Alienauroa* (Lissovsky, 2014; Wang et al., 2020). The phylogeny and taxonomy of pikas have been the subject of much attention during the last decades. However, many phylogenetic

studies of *Ochotona* have been based on single loci (Lanier & Olson, 2009; Lissovsky, 2014; Niu et al., 2004; Yu et al., 2000) or have lacked taxa, and only a few studies have been based on multilocus data (Koju et al., 2017; Melo-Ferreira et al., 2015; Wang et al., 2020).

The number of pika species fluctuate between studies, but tend to increase from 25–30 species in early studies (Hoffmann & Smith, 2005; Lissovsky, 2014; Smith et al., 1990) to 29–34 species in more recent studies (Smith et al., 2018; Wang et al., 2020). This increase is largely

due to the incorporation of molecular and/or morphological data that have revealed considerable cryptic diversity, and the elevation of several taxa previously treated as subspecies to species level (Dahal et al., 2017; Koju et al., 2017; Lissovsky, 2014; Lissovsky et al., 2007, 2019; Liu et al., 2017; Wang et al., 2020).

Afghan pika *Ochotona rufescens* (Grey, 1842) is one of the members of the genus *Ochotona*, which is distributed in Western and Central Asia. The range of this species extends from the Zagros Mts and the northeast Alborz Mountain chain in Iran, to the Kopet Dagh Mountains, the Paropamisus Mountains in Afghanistan and mountain ranges of western Pakistan (Corbet, 1978; Ellerman & Morrison-Scott, 1951; Hassinger, 1973; Hoffmann & Smith, 2005; Lissovsky, 2016). Traditional taxonomy of *O. rufescens* recognized three subspecies (Ellerman & Morrison-Scott, 1951) mainly based on external and cranial characters and pelage colour. However, not all subspecies defined on morphology could be differentiated in a study revisiting morphological characters (Mohammadi et al., 2018), as original descriptions of taxa were made on the basis of qualitative characters without sufficient accounting for seasonal and local pelage coloration variation (Mohammadi et al., 2018).

The type series of Afghan pika *O. r. rufescens* (Grey, 1842) was collected from the vicinity of Kabul, Afghanistan. Subsequently, several subspecies have been proposed. Thomas (1911), described *O. r. regina*, Thomas, 1911 from Kopet Dagh, west of Ashkhabad, Turkmenistan, based on its generally larger body and skull size, as well as the smaller and more colourful *O. r. vizier* Thomas, 1911 from Koh-rud, Central Iran, the latter mentioned already by Blanford (1876). *Ochotona rufescens shukurovi* Heptner, 1961 was described from the Great Balkhan Mountains, Turkmenistan, but was synonymized with *O. r. regina* by Mohammadi et al. (2018) based on mitochondrial phylogeny. The intermediate-sized *O. r. vulturna* Thomas, 1920 is known from Chiltan Mountains in Quetta, Pakistan. The validity of the taxon was questioned by Ellerman and Morrison-Scott (1951), who considered it an aberrant form based on pelage characters. Lissovsky (2014) considered its morphology as within the normal variation of *O. rufescens* with regard to cranial shape.

Despite efforts to elucidate the taxonomic status of different populations of *O. rufescens* based on morphological characters (Aliabadian, 1995; Fostowicz-Frelik et al., 2010; Mohammadi et al., 2018) and morphometric analyses (Lissovsky, 2014; Mohammadi et al., 2018), the taxonomic status of some populations remain unresolved. Presumably stabilizing selection in the mountainous habitats occupied by Afghan Pikas is conserving morphometric features, thus resulting

in minimal morphological divergence in *O. rufescens* (Mohammadi et al., 2018).

In this survey, we estimated a time-calibrated multilocus coalescent-based phylogeny based on mitochondrial *cyt b* and six nuclear loci to assess the taxonomic relationship of different taxa within *O. rufescens* (*rufescens*, *regina*, *vizier*, *shukurovi*) in Western Asia.

## 2 | MATERIAL AND METHODS

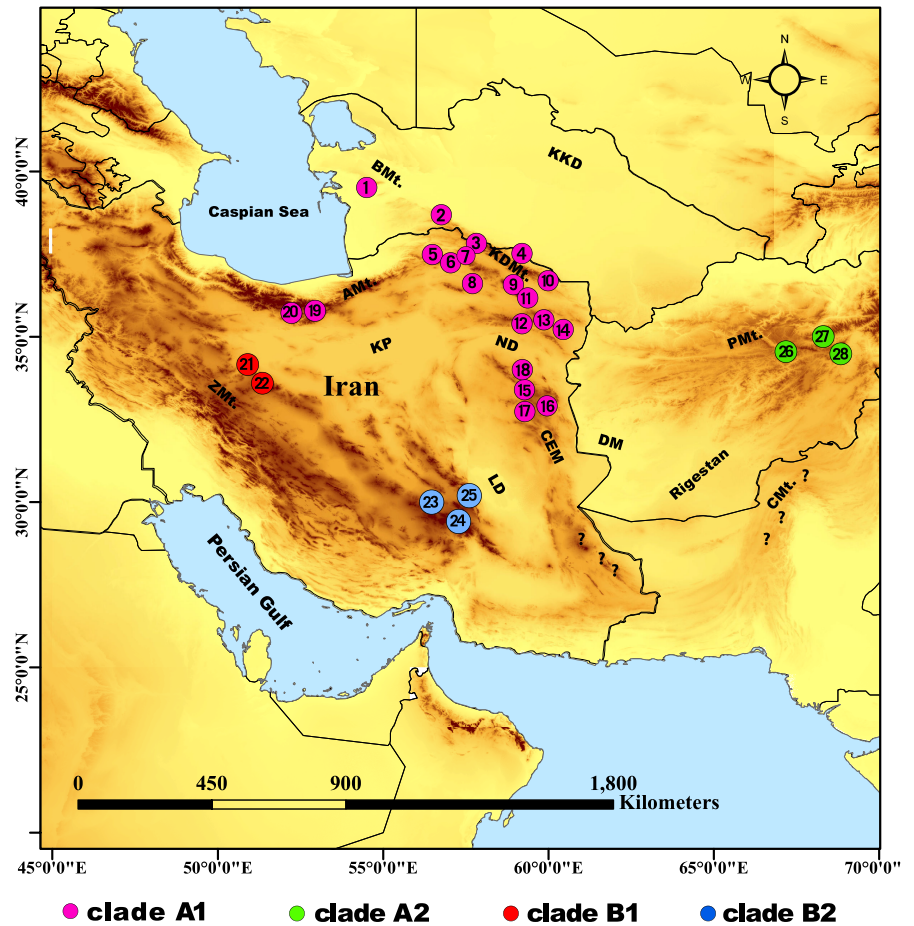
### 2.1 | Sampling strategy

We used 37 samples of *O. rufescens* from a previous study (Mohammadi et al., 2018) and 17 new samples from additional localities in Iran and Afghanistan. We also retrieved 37 *O. rufescens* cytochrome *b* (*cyt b*) sequences from GenBank. Efforts to provide samples from Afghan pikas previously reported by Čermák et al. (2006) in Taftan on the Pakistan border, southeast Iran failed. In total, the mitochondrial *cyt b* of 91 samples of *O. rufescens* from different localities of Western Asia were used in this study: Iran (82 specimens), Afghanistan (four specimens) and Turkmenistan (five skin samples). To provide a phylogenetic framework for the *O. rufescens* phylogeny, we also obtained six tissue samples from different species of pikas (*O. princeps*, *O. hyperborea*, *O. thibetana*, *O. curzoniae* and *O. gloveri*) from the Burke Museum of Natural History, University of Washington, Seattle, USA [UWBM] (Figure 1, Tables S1 and S2). Additionally, sequences of pikas were retrieved from GenBank and used for phylogenetic analyses (Table S2). *Lepus europaeus*, *L. capensis*, *L. granatensis* and *Oryctolagus cuniculus* were used as outgroups (not shown in the trees in the text; see Table S2).

### 2.2 | Laboratory procedures and sequencing

DNA was extracted from kidney and muscles preserved in 95% ethanol using the QIA Quick DNEasy Kit (Qiagen, Inc), following the manufacturer's instructions. The *cyt b* gene was amplified and sequenced according to Mohammadi et al. (2018). We sequenced six nuclear markers (IL1RAPL1, SPTBN1, TSHB, PRKCI, UCP2 and UCP4) for specimens of the *O. rufescens* species complex (15 to 27 specimens; Table S2) and for the specimens obtained from the Burke Museum. The PCR conditions, primers used for amplification and sequencing of nuclear loci are listed in Table S3. All amplification products were purified with ExoTap kit (Werle et al., 1994) and then sequenced in two reactions with forward and reverse primers. All sequences were deposited in GenBank (Table S2).

**FIGURE 1** Map of collecting localities and distribution of lineages for *Ochotona rufescens* in the Western and Central Asia. Clades are highlighted with different colours: red, Central Iran; green, Afghanistan; blue, Kerman, southeast Iran; pink, Kopet Dagh/ Great Balkhan. Numbers indicate the sampling localities mentioned in Table S1. Abbreviations: AMt. (Alborz Mountains); ZMt. (Zagros Mountains); CEM (Central East Mountains); KDMt. (Kopet Dagh Mountains); BMt. (Great Balkhan Mountains); PMt. (Paropamisus Mountains); CMt. (Chiltan Mountains); KP (Kavir Plain); LD (Lut Desert); ND (Namak Desert); DM (Dasht e Margo); KKD (Kara Kum Desert)



### 2.3 | Alignment and data sets

Sequences were aligned, edited and quality trimmed using MegAlign 4.03 in the DNASTAR package (DNASTAR Inc.). Consensus sequences for each specimen were created from sequences in forward and reverse directions using the DNASTAR package. Pairwise sequence distance was calculated by Kimura-2-parameter (K2P) distances in the MEGA X (Kumar et al., 2018). We analysed the data set in five combinations—(1) single locus analyses of each individual mitochondrial and nuclear marker, (2) all mitochondrial (*cyt b*) and nuclear markers (IL1RAPL1, SPTBN1, TSHB, PRKCI, UCP2 and UCP4) concatenated, (3) nuclear markers (IL1RAPL1, SPTBN1, TSHB, PRKCI, UCP2 and UCP4) concatenated, (4) mtDNA (*cyt b*) and all six nuDNA (IL1RAPL1, SPTBN1, TSHB, PRKCI, UCP2 and UCP4) to generate a coalescent-based Bayesian multi-locus species tree and (5) six nuclear markers (IL1RAPL1, SPTBN1, TSHB, PRKCI, UCP2 and UCP4) to generate a coalescent-based Bayesian multilocus species tree.

### 2.4 | Phylogenetic analysis

Data sets 1, 2 and 3 were first analysed using a Bayesian inference (BI) in BEAST version 2.2.1 (Bouckaert

et al., 2014) and then all data sets were analysed separately using maximum-likelihood (ML) methods in PAUP 4.0b10 (Swofford, 2003). The choice of substitution model was determined based on the Bayesian information criterion (BIC; Schwarz, 1978) and akaike information criterion (AIC; Akaike, 1974) as implemented in jModeltest 2.1.1 (Darriba et al., 2012) and PartitionFinder (Lanfear et al., 2012, 2017; Table S4). The BI analysis was run for 100 million generations, with default priors. We sampled parameters and tree topology every 10,000 generations and discarded the first 25% of trees as burn-in. Trees were summarized using TreeAnnotator version 2.2.1 (Rambaut & Drummond, 2015) and displayed in FigTree 1.4.3 (Rambaut, 2016). Posterior probability (PP) values higher than 0.95 were considered strong support for each clade. ML analyses were carried out under heuristic tree search with ten random addition sequence replicates and tree bisection reconnection (TBR) branch swapping. To assess support for internal nodes, we ran nonparametric bootstrapping (500 replicates; Felsenstein, 1985) under ML, with a single random addition sequence replicate per bootstrap replicate on the CIPRES Science Gateway (Miller et al., 2010). Maximum-likelihood bootstrap (BP)  $\geq 60\%$  was considered strong support.

## 2.5 | Divergence time based on coalescent-based species tree

The divergence times for data sets 4 (cyt *b* and six nuclear loci) and 5 (six nuclear markers) were calculated using the coalescent species tree estimation model implemented in \*BEAST (Bouckaert et al., 2014). We used the data set from Melo-Ferreira et al. (2015) consisting of the cyt *b* and six nuclear loci (IL1RAPL1, SPTBN1, TSHB, PRKCI, UCP2 and UCP4) and added our new data for a total of 49 individuals (28 new sequences + 21 sequences from Melo-Ferreira et al., 2015) of 15 lineages of the genus *Ochotona* (Table S2). We delimited 'species' based on results from the cyt *b* analyses. We used an uncorrelated lognormal relaxed clock, Yule tree prior and a random starting tree. We ran the final analyses with 100 million generations in BEAST, sampling every 10,000 generations. See above for further details.

Four different calibration dates for mean height of the divergence between Ochotonidae and Leporidae were used for estimation of divergence time. Three of these calibration dates: 31 Mya (Matthee et al., 2004), 37 Mya (Asher et al., 2005; McKenna & Bell, 1997) and 65 Mya (Bininda-Emonds et al., 2007), were used following Lanier and Olson (2009) and Melo-Ferreira et al. (2015), but we also applied 52 Mya according to Koju et al. (2017).

## 3 | RESULTS

### 3.1 | Molecular data

In total, 140 new sequences from six nuclear loci (IL1RAPL1, SPTBN1, PRKCI, TSHB, UCP2 and UCP4) and one mitochondrial protein-coding gene (cyt *b*) for 60 individuals of the genus *Ochotona* including *O. rufescens*, *O. princeps*, *O. hyperborea*, *O. thibetana*, *O. curzoniae* and *O. gloveri* were included. Our data sets had

total lengths of 1138 bp of mitochondrial cyt *b* sequences except 12 specimens with only 32.2% missing data (366 out of 1138), and 3425 bp of six nuclear markers including IL1RAPL1 (825 bp), SPTBN1 (708 bp), PRKCI (406 bp), TSHB (466 bp), UCP2 (412 bp) and UCP4 (608 bp). In total, the alignment was 4563 bp (Table S5).

### 3.2 | mtDNA phylogeny of the genus *Ochotona*

The mitochondrial cyt *b* tree (data set 1) based on BI and ML for the genus *Ochotona* included 60 new samples from six species (*O. rufescens*, *O. princeps*, *O. hyperborea*, *O. thibetana*, *O. curzoniae* and *O. gloveri*) as well as 79 sequences retrieved from GenBank (Tables S1 & S2), and showed the existence of several clades and subclades clustered within five major clades or lineages (Figure S1) consistent with the five subgenera *Ochotona*, *Pika*, *Conothoa*, *Alienauroa* and *Lagotona*. *Ochotona pusilla* (*Lagotona*) was placed as sister to the rest of four clades.

The monophyly of the subgenus *Conothoa*, which includes Afghan pikas, was strongly supported by the cyt *b* phylogeny. The phylogenetic analysis of the Afghan pikas (91 samples) recovered two well supported (PP 1.00, BS 99) and deeply diverged clades (A-B; Figure 2). These major clades were further subdivided into four geographically structured subclades: subclade (A1) included specimens from northern Iran (Central Alborz, Kopet Dag, Northeastern Iran) and southern Turkmenistan (including specimens from the type localities of *O. r. regina* and *O. r. shukurovi*); subclade (A2) included specimens from Afghanistan (including specimens collected near the type locality of *O. rufescens*); subclade (B1) included specimens from the Kerman area, south Iran (*O. rufescens* cf *vizier*); and subclade (B2) included specimens collected from Kohrud mountains (point 22, Figure 1: Lat 33.59N and Lon 51.36 E) near the Ghohrud village, Kashan, Central Iran,

	A1	A2	B1	B2	A1	A2	B1	B2
cyt <i>b</i> \PRKCI					UCP4\SPTBN1			
A1	<b>0.6–0.1</b>	1.1	1.3	-	A1	<b>0.1–0.2</b>	0.6	2.6
A2	3.3	<b>1.1–0.5</b>	0.9	-	A2	0.2	<b>0.1–0.1</b>	2
B1	5.4	5.5	<b>0.2–0</b>	-	B1	0.6	0.6	<b>0–0</b>
B2	6.4	7.1	4.2	<b>0.2</b>	B2	-	-	-
TSHB\UCP2					IL1RAPL1			
A1	<b>0–0.2</b>	0.9	0.8	-	A1	<b>0.1</b>		-
A2	0.2	<b>0–0.3</b>	1.0	-	A2	0.3	<b>0.1</b>	-
B1	0.7	0.4	<b>0–0</b>	-	B1	0.4	0.2	<b>0</b>
B2	-	-	-	-	B2	-	-	-

**TABLE 1** Genetic distance (K2P) among the lineages calculated based on cyt *b* and six nuclear markers, implemented in MEGA program using K2P method (the numbers above bold diagonal indicated the genetic distances between clades for the right locus in the table and the numbers below diagonal indicated the genetic distances between clades for the left locus in the table)

Note: Values indicating intra-lineage distances are shown in bold.

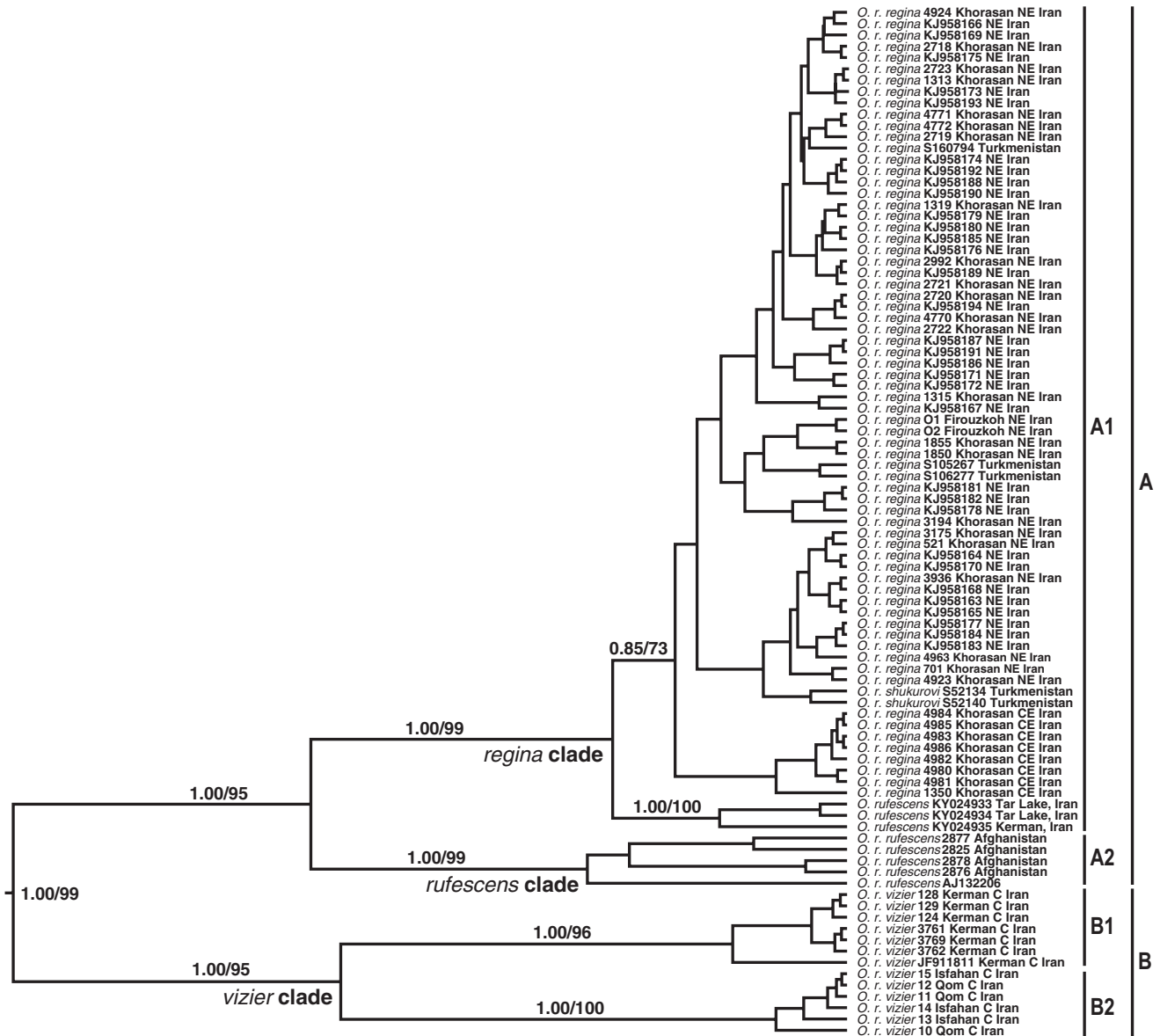


FIGURE 2 Phylogenetic tree of *Ochotona rufescens* obtained by Bayesian analysis of the *cyt b* data set (1138 bp). Bayesian posterior probabilities ( $\geq 0.85$ ) and ML bootstrap values ( $\geq 70\%$ ) are indicated on the branches, respectively. The outgroups are not shown here but see Figure S1.

approximately 7 km from the inferred location of the type locality of subspecies *O. r. vizier*. The support for the topology was very high (PP 1.00; BS 95). The highest genetic distance between the two major lineages related to clades A2 (*O. r. rufescens*) and B2 (*O. r. vizier*) which is 7.1% (Table 1).

### 3.3 | Divergence time based on coalescent-based species tree

The major divergences within the genus *Ochotona* began during the Early to Middle Miocene (node B: 6.6–12.49 million years ago [mya]; Figure 4a,b & Table 2), based on mito-nuclear loci and nuclear loci (data sets 4 and 5)

inferred from \*BEAST analyses with different calibration points. The diversification of the subgenus *Conothoa* (node F in Figure 4a,b) occurred during the Late Pliocene to Early Miocene, with an estimated divergence time of 8.07 to 3.57 mya (Table 2). Also, the time of divergence between the *O. rufescens* lineages A and B (Figure 4, node H, PP 1.00) is estimated to about 1.43 to 0.58 Mya during the Pleistocene.

### 3.4 | Phylogeny of nuclear DNA and concatenated data sets

Three of the phylogenetic inferences based on single-locus analyses of the nuclear loci (data set 1), SPTBN1,

**TABLE 2** Comparison of different calibration points (five calibration points) of Leporidae-Ochotonidae divergence for estimating of divergence time of the genus *Ochotona* inferred of \*BEAST based on seven (mtDNA and nuDNA) and six (nuDNA) markers

Node (A)	B	C	D	E	F	G	H	I	J	K	L	M	N	O
6 loci														
<b>Calibration 31</b>	6.6	6.26	1.24	0.72	4.4	3.05	0.58	3.86	2.67	1.28	1.07	0.15	0.61	0.49
HPD 27.4–34.49	5.42–7.89	5.08–7.69	0.72–1.83	0.22–1.28	3.53–5.43	2.39–3.76	0.31–0.9	3.07–4.65	1.92–3.4	0.84–1.62	0.64–1.42	0.02–0.3	0.37–0.85	0.27–0.76
<b>37</b>	7.27	6.89	1.37	0.94	4.88	3.31	0.66	4.26	2.94	1.28	1.12	0.14	0.74	0.59
HPD 31.96–40.48	6.45–8.44	5.97–7.89	0.84–1.79	0.33–1.53	3.9–6	2.49–4.17	0.36–0.96	3.12–5.52	2.13–3.75	0.7–1.55	0.93–1.64	0.01–0.28	0.5–1.03	0.31–0.89
<b>52</b>	10.28	9.72	1.75	1.19	6.78	4.72	0.94	6.26	4.27	1.95	1.69	0.24	0.99	0.8
HPD 45.94–57.92	8.72–11.8	8.23–11.24	1.07–2.72	0.27–2.35	5.47–8.22	3.57–5.9	0.52–1.4	4.82–7.61	3.05–5.62	1.4–2.59	1.12–2.33	0.02–0.5	0.64–1.39	0.42–1.19
<b>65</b>	12.49	11.69	2.32	1.52	8.07	5.58	1.11	7.1	4.88	2.21	1.98	0.27	1.17	0.94
HPD 56.5–71.5	10.95–14.35	9.94–13.56	1.52–3.32	0.57–2.88	6.59–9.54	4.4–6.7	0.65–1.66	5.74–8.37	3.69–6.05	1.62–2.83	1.38–2.6	0.02–0.56	0.78–1.6	0.48–1.38
7 loci														
<b>31</b>	5.73	5.39	1.55	1.29	3.57	2.82	0.69	4.09	2.61	1.67	1.47	0.21	0.99	0.88
HPD 26.87–32.09	5.12–6.39	4.72–6.03	1.19–1.9	0.91–1.61	3.14–4.02	2.41–3.22	0.52–0.86	3.31–4.73	2.09–3.06	1.32–1.98	1.17–1.75	0.06–0.38	0.75–1.21	0.7–1.1
<b>37</b>	7.05	6.46	2.04	1.64	4.48	3.57	0.86	4.76	3.16	1.95	1.77	0.23	1.26	1.14
HPD 33.38–38.36	6.34–7.91	5.73–7.23	1.51–2.5	1.12–2.32	3.9–5.18	2.97–4.08	0.56–1.11	3.82–5.53	2.68–3.72	1.67–2.21	1.46–2	0.07–0.41	0.85–1.65	0.83–1.48
<b>52</b>	9.85	9.27	2.8	2.4	6.37	4.96	1.22	6.81	4.38	2.83	2.56	0.35	1.74	1.55
HPD 48.12–53.72	9.03–10.71	8.26–10.21	2.39–3.23	1.88–2.92	5.47–7.39	3.93–5.97	0.9–1.53	5.84–7.9	3.68–5.28	2.17–3.56	1.95–3.21	0.08–0.61	1.32–2.13	1.15–1.96
<b>65</b>	12.28	11.56	3.42	3.07	8	6.12	1.43	8.59	5.31	3.48	3.11	0.48	2.12	1.89
HPD 60.72–68.17	10.89–13.82	10.58–12.92	2.73–4.06	2.4–3.95	6.99–8.99	5.37–6.91	1.04–1.94	7.64–9.57	4.31–6.49	2.79–4.29	2.46–3.67	0.16–0.82	1.58–2.71	1.26–2.45

Note: The nodes calibrated with five estimated divergence times are shown in Figure 4.

UCP2 and UCP4, recovered clades A and B1 as sisters (B2 was not considered because no nuclear sequences were available for this clade), whereas IL1RAPL1, PRKCI and TSHB recovered the members of clade B1 as monophyletic but nested within clade A (Figure S2). Nearly identical topologies were obtained in both the phylogenetic reconstruction based on the concatenated sequences of seven markers (one mitochondrial and six nuclear loci; data set 2) with 4563 bp, and the concatenated sequence of six nuclear loci with 3425 bp (data set 3; Figure 3a,b), supporting clades A and B as sisters. Phylogenetic analyses based on both concatenated data sets (data sets 2, 3; Figure 3a,b) also supported the presence of two long separated allopatric clades within Afghan pikas (clades A and B; Figure 3a,b) with high posterior probability (PP = 1.00, MLB 100). In concordance with the mitochondrial *cyt b* tree (Figure 2 and Figure S1), phylogenetic analyses of the concatenated sequence matrix (Figure 3a,b) also demonstrates relatively high genetic

divergence between subclades A1 from Kopet Dagh-Central Alborz and A2 from Afghanistan.

## 4 | DISCUSSION

### 4.1 | Phylogeny

The phylogeny of the genus *Ochotona* is overall well resolved and well supported (Figures 3 and 4, Figures S1 and S2). The topologies and node support of the multi-locus phylogenies based on seven concatenated markers (*cyt b* and six nuclear loci) and six nuclear loci, respectively, were nearly similar with minor differences (Figure 3). However, the individual performance of the different nuclear loci differed slightly in reconstruction of the relationships within Afghan pikas. Visual inspection of the phylogenies reveals that there were no strongly supported conflicts either between the single locus analyses

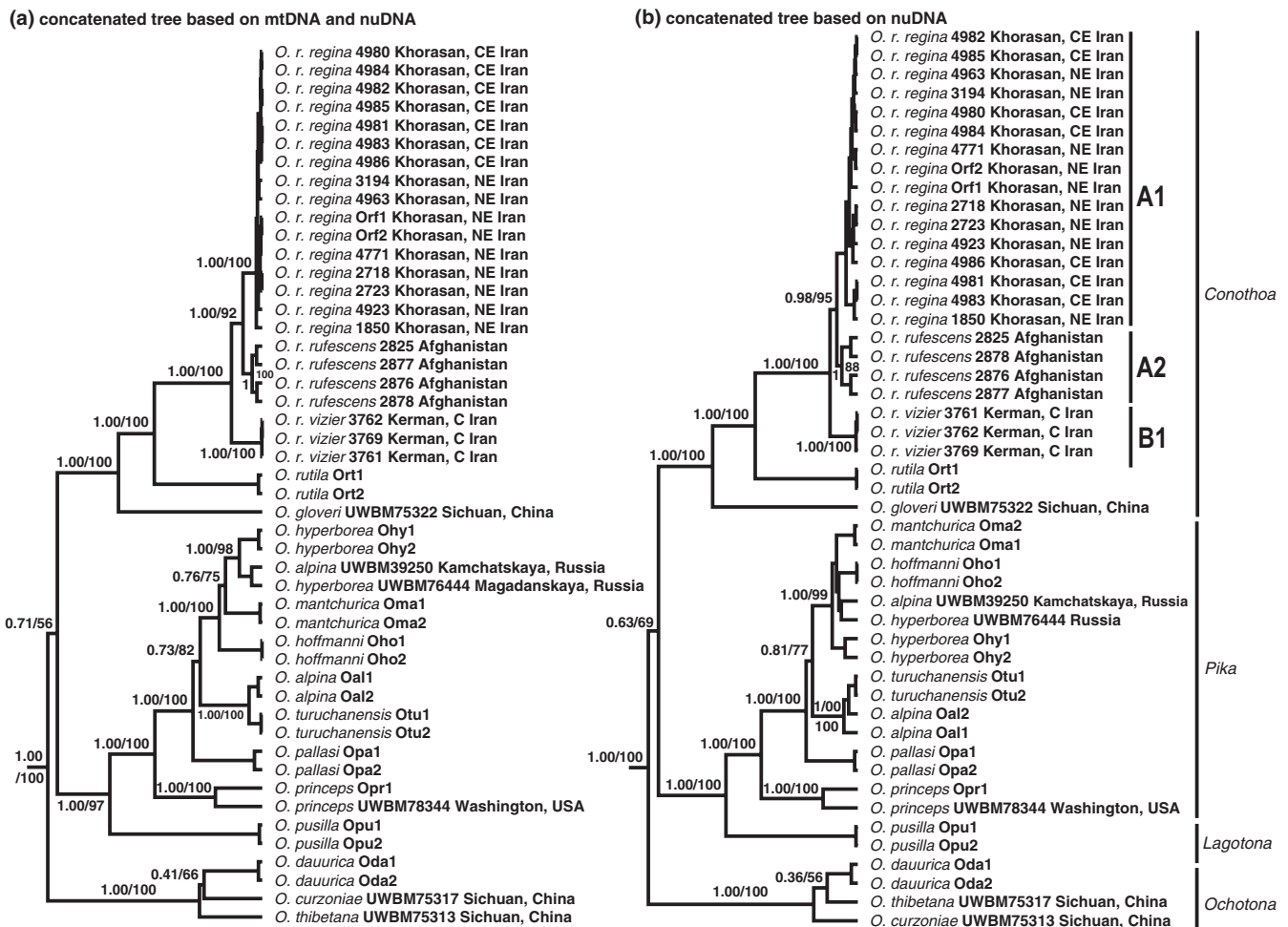


FIGURE 3 Phylogenetic concatenated trees based on a) six nuclear and a mitochondrial *cyt b* data sets (4563 bp) and b) six nuclear data sets (3425 bp). Phylogenetic tree of the genus *Ochotona* lineages obtained by BI of the concatenated *cyt b* + PRKCI + TSHB + UCP2 + UCP4 + SPTBN1 + IL1RAPL1 data set. Bayesian posterior probabilities ( $\geq 0.90$ ) are indicated above branches. The outgroups are not shown here but see Figure S2.

of nuclear loci (Figure S2) or with the mitochondrial phylogeny (Figure 2).

We found a deep divergence between two well-differentiated lineages within Afghan pikas (Figures 3 and 4) separating lineages from central and southern Iran (clade B, Figure 2) from the other lineages (clade A). There is no geographical overlap between these two lineages (Figure 1). The deep divergence of *O. r. vizier* from Central Iran, evident in the mtDNA phylogeny, in combination with its non-overlapping distribution, suggests long standing isolation from populations in clade A. We hypothesized that the Central Iran population (clade B) diverged from the other populations (clade A) following desertification of the Lut Desert (LD, Figure 1) and the Kavir Plain (KP, Figure 1; Motamed, 1994; Kehl, 2009) and appear to have remained isolated since then. Consequently, gene flow appears to have ceased or become severely impaired by the formation of the barriers posed by the Lut and Kavir Plains, thus initiating the divergence of the ancestor of *O. rufescens*. This inferred isolation by desert barriers is hypothesized to have promoted complete lineage sorting in mtDNA and in 3 out of 6 nuclear loci between Central Iran population and other populations of Afghan pikas. This long standing vicariance event in combination with morphological diversification, suggests that promotion of *O. r. vizier* to species level is warranted (Figure S2), and we will treat it as such in the following.

In contrast, *O. r. shukurovi* is nested in the *O. r. regina* clade, based on the mitochondrial marker (Figure 2). The separation between populations of Kopet Dagh and Great Balkhan thus seems to be not long-lasting. As mentioned in Mohammadi et al. (2018) separation of these two populations may be related to the last transgression of the Caspian Sea dated back to 70–40 kya and also post-Pleistocene flow of the Amu Darya distributary channel to the Caspian Sea.

The phylogenetic position of Afghan pikas occurring in Taftan, as well as of *O. r. vulturina* has not been studied by molecular data and needs to be investigated. Also, the exact boundaries of the range of this taxon need to be determined. Moreover, the populations of Afghan pikas from different localities in Afghanistan are clearly diverged in the mitochondrial phylogeny and are recovered as monophyletic by some of the nuclear markers, suggesting a need for further study (Mohammadi et al., 2018).

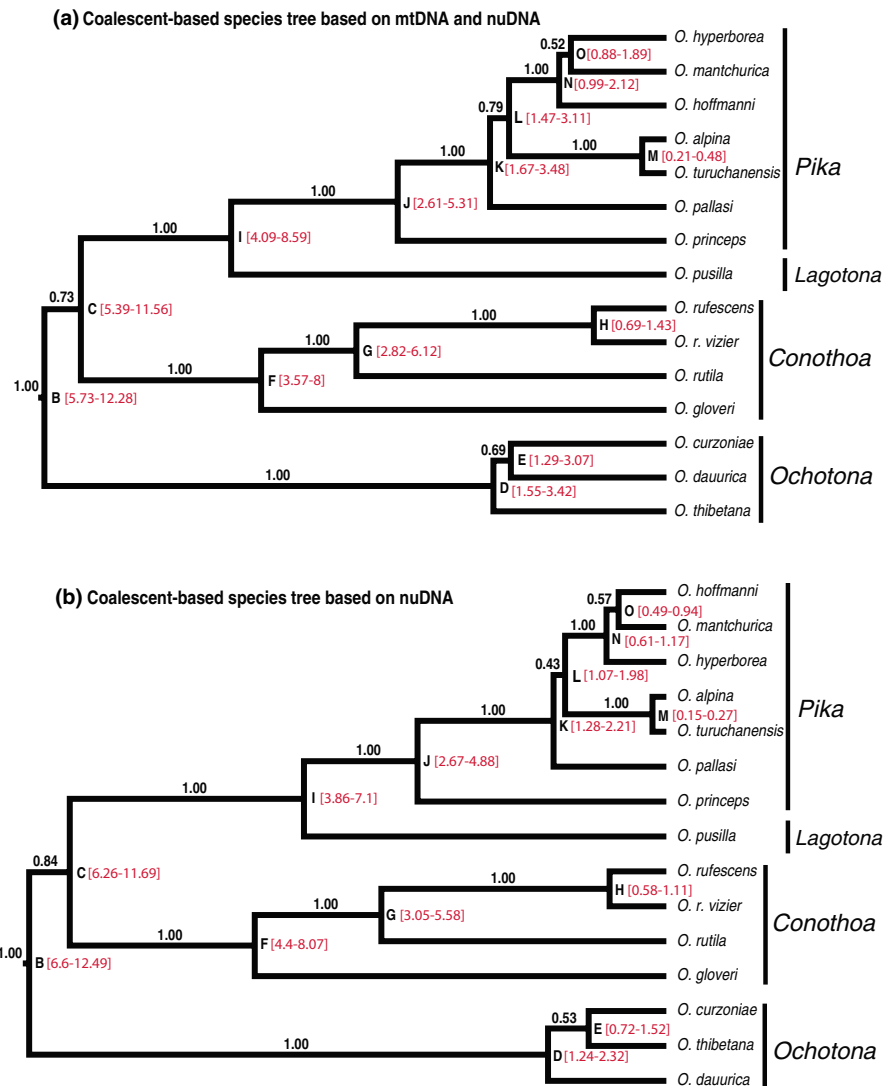
Additionally, the sequence (KY024935; Koju et al., 2017) emanates from Kerman (Central Iran) according to the supplemental information in the publication. However, this information is based on a misprint as the specimen MVZ198794 is from Tehran Province according to the museum label (Figure 2).

## 4.2 | Divergence time and distributional range

Wang et al. (2020) proposed that pikas originated at high altitudes on the Qinghai Tibetan Plateau and that *O. rufescens* originated from a population dispersing from that area in late Miocene or early Pliocene. According to Wang et al. (2020), the ancestor of this population was adapted to high altitudes and cool habitats. The pika distribution patterns that subsequently formed in Western and Central Asia may have been strongly affected by recurrent fluctuations between periods of warmer climate, with suitable habitat mostly available only at the highest altitudes, but during periods of cooler climate, suitable habitat may sometimes have become available across lowlands between mountains. During interglacials, the lower altitudes likely became unfavourable, causing Afghan pikas to again retreat to higher elevations, and marginal populations to go extinct (Mohammadi et al., 2018). The molecular divergence estimation for two data sets of the seven mitochondrial and nuclear loci, and the six nuclear loci, respectively, based on calibration points from fossil evidence, suggest that *O. vizier* in Central Iran diverged from other populations of Afghan pikas approximately 0.58–1.43 million years ago (mya; 95% HPD = 0.31–1.94 mya; Figure 4, Table 2), perhaps as a result of reaching new areas in connection with the late Calabrian ice age. Paleontological estimation of the age of the oldest reported fossil record of Afghan pikas in Western Asia is Middle Pleistocene sites (0.8 to 0.13 mya) from Sel-Ungur, Kyrgyzstan (Averianov, 2001), which would be after or near the time of the split between clade A and clade B (Figure 2). During the early Pleistocene arid conditions expanded in Western and Central Asia and resulted in formation of deserts in the Central and Eastern parts of Iran (Koufos et al., 2005; Wang et al., 2016), probably causing the ancestral forms of Afghan pikas to become trapped on 'sky islands' of different mountains of Iran, for example in Zagros, Alborz and mountains of Central Iran. Climate oscillations during Pleistocene, when the temperature decreased, may have developed suitable conditions during glacials to allow some populations of Afghan pikas to shift their ranges to lower altitudes and disperse across valleys to find new habitats. However, these hypothetical opportunities for dispersal were apparently not sufficient for providing contact between the emerging clades A and B (Figure 2) and the range of *O. vizier* seems to have remained isolated. In addition, the onset of glacial periods were followed by a growing desert belt in Western Asia (Dodonov, 1991) and expansion of landscapes dominated by C4-grass (Akhani et al., 1997; Akhani & Ziegler, 2002; Winter, 1981) which is unfavourable as food for pikas (Ge et al., 2012, 2013). All this in combination presumably



**FIGURE 4** Species trees of the genus *Ochotona* inferred of \*BEAST analysis based on (a) mtDNA + nuDNA data set (4563 bp) and (b) nuDNA data set (3425 bp). Inferred mean node ages calibrated with five estimates of Leporidae–Ochotonidae divergence (node A; not shown on the trees) and the 95% highest posterior density (HPD) intervals are also shown in Table 2. The red numbers at nodes represent the divergence times between lineages (details given in the Table 2). Bayesian posterior probabilities ( $\geq 0.90$ ) are indicated above branches. The outgroups are not shown here but see Figure S2.



favoured ecological isolation and upheld vicariance between different populations of Afghan pikas. Sampling and molecular results thus indicated *O. vizier* dispersal opportunities to have been restricted by deserts distributed in the Central Iranian Plateau, for example Maranjab Desert and Namak (Salt) Lake in the north, Mesr Desert, Kavir Plain in the east, also Mehriz and Ardakan Deserts. Additionally, the salt lowland plains extending from southern foothills of the Alborz Mountains to the central Iranian Plateau provided strong barriers between *O. r. regina* in sky islands of Central Alborz and Kopet Dagh and *O. vizier* which caused vicariance. Presumably, *O. vizier* had been trapped in sky islands served by interspersed mountains within Central Iranian Plateau surrounded by deserts. Moreover, the species *O. vizier* diverged into two main lineages B1 and B2 which are in turn separated by Mehriz, Ardakan and Mesr Deserts. Extension of Lut, Tabas and Polond Deserts in the east also provide impenetrable barriers between *O. vizier* in Iran and *O. r. rufescens* in Afghanistan and may also restrict gene flow.

### 4.3 | Morphological variation

The *O. rufescens* lineages in Western and Central Asia formed geographically structured groups, but this pattern of molecular divergence was not paralleled by corresponding morphometric divergence and not entirely compatible with previously recognized taxonomic boundaries. Morphometric analyses, including the type specimens of *O. r. rufescens*, *O. r. regina*, *O. r. vizier*, *O. r. shukurovi* and *O. r. vulturina*, demonstrated no variation in morphometric characters among Afghan pikas (Mohammadi et al., 2018), which can be a result of adaptive pressure related to behaviour, lack of divergent selection, high degree of homoplasy, and adaptive divergence in skull shape (Bickford et al., 2007; Dahal et al., 2017; Egea et al., 2016; Feijó et al., 2020; Kraatz & Sherratt, 2016) or strong stabilizing evolution (Mohammadi et al., 2018; Yu et al., 2000). The lack of morphometric diversity in Afghan pikas in external and cranial measurements make taxonomic decisions based on these alone difficult. In addition, pelage

colour has been shown to vary both in relation to age and season and may not be as taxonomically meaningful as sometimes believed (Ognev, 1940).

In summary, according to our data the *O. r. regina* haplogroup is distributed from Great Balkhan in Turkmenistan, south to the Kopet Dagh Mountains, extending to central east Iran to Bagheran Mountains in Southern Khorasan. Our molecular results suggest that taxonomic recognition of *O. r. shukurovi* is not justified from a phylogenetic perspective, whereas the taxonomic status of *O. r. vulturna* could not be evaluated by this study. Moreover, we have no evidence of the *O. r. rufescens* haplogroup occurring outside Afghanistan, although more sampling is needed to determine its exact range. Central Iran is occupied by the *O. vizier* haplogroup, so far recorded from Kerman in the east and the Kuhrud Mountains in the central west (Figure 1). The holotype specimen of *O. vizier* (Natural History Museum [London] no. NHM 74.11.21.5) was collected by Blanford at Kohrud, Central Iran and described as a new subspecies of *O. rufescens* by Thomas (1911). The type locality is given as Koh-rud north of Isfahan, Central Persia, at an altitude of 9000 feet, but there is some confusion as to exactly where this place is located, as alternative transliterations are possible and do not all converge on the same place. Blanford describes the location where he collected four specimens in July 1872 as the pass where the road crosses over the mountain range near the village of Kohrud. According to Blanford's description of his voyage, particularly in his account of the geology of the area (pp 499–500, Blanford, 1876), the most likely location of this pass appears to be 33.62°N, 51.43°E (33°37'19.92"N, 51°25'37.19"E), at 2837 m asl, just south of the village now spelt Ghohrud.

## 5 | CONCLUSION

Climate fluctuation and ecosystem instability during Pleistocene probably caused Afghan pikas to frequently shift their ranges between higher and lower altitudes. During periods of cold climate favouring steppe, pikas may have been able to descend to the foothills and disperse across lower altitudes if the edaphic conditions permitted, facilitating gene flow between populations. On the other hand, when either humid and warm or hot and dry conditions predominated, Afghan pikas were forced to withdraw to the highest elevations.

We did not find any shared mitochondrial haplotypes between the two different major clades A and B (Figure 2), supported by similar structuring in some of the nuclear genes (Figure S2). All loci recovered clade B as monophyletic, which indicates strict isolation of pikas on different

'sky islands' during warm periods and that clades A and B are isolated, and we therefore propose that *O. vizier*, although cryptic, is recognized as a species on the basis of long-standing reproductive isolation from populations of *O. rufescens*. The evidence further suggests that migration of Afghan pikas during periods of cooling, when conditions hypothetically allowed for dispersal, seem to have been rare and stochastic events, probably due to the hostile edaphic condition of lower intervening areas between mountain ranges. Therefore, in spite of the fact that the high mountain ranges of the Alborz and eastern Zagros act as refugia for pikas during warm periods, the desert habitats surrounding them appear to rarely have permitted allopatric populations of Afghan pikas to have genetic exchange, rendering the usual understanding of the concept of refugia somewhat ambiguous in this case, as they are just as much trapped in their habitat as taking refuge there.

This study of a mountain-dwelling mammal in the context of unique edaphic conditions of Western and Central Asia, sheds light on the impact of Pleistocene climate fluctuations on cold adapted organisms, which may be forced into refugia during the warmer climates of interglacials, rather than conforming to the norm of enduring in refugia during the ice ages.

## ACKNOWLEDGEMENTS

We gratefully thank the officials and staff of the Department of Biology and Environmental Sciences, Systematics and Biodiversity at University of Gothenburg. Permission to collect was authorized by the Iranian Department of Environment (Permission Number: 93/53381; 2015; 25th January).

## FUNDING INFORMATION

This work was supported by the Swedish Research Council (grant No. 2015-04651).

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**How to cite this article:** Mohammadi, Z., Ghorbani, F., Aliabadian, M., Lissovsky, A. A., Yazdani Moghaddam, F., & Olsson, U. (2022). Multilocus phylogeny reveals habitat driven cryptic diversity in *Ochotona rufescens* (Ochotonidae). *Zoologica Scripta*, 51, 617–628. <https://doi.org/10.1111/zsc.12564>