Journal of Wildlife and Biodiversity 1(1): 12-18 (2017)

by Arak University, Iran (http://jwb.araku.ac.ir/)

Research Article

Assessment of the Phylogenetic Status of Afghan Pika (*Ochotona rufescens)*

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Received: 5 July 2017 / Revised: 8 August 2017 / Accepted: 14 August 2017 / Published online: 18 August 2017. Ministry of Sciences, Research and Technology, Arak University, Iran.

Abstract

Phylogenetic relationships between Afghan Pika (*O. rufescens*) and others in the world are still unclear. Morphological phylogeny study of the genus *Ochotona* by different experts showed the different taxonomic position of the Afghan Pika, but could not determine the taxonomic position of this species. Previous phylogenetic studies based on mtDNA markers showed the different sister groups of the species. In this study, we aimed to unveil the phylogenetic relationship of the Afghan Pika, adding more specimens to the phylogenetic trees. In total, 355 different sequences relating to different pikas were retrieved from GenBank and 25 individuals from Iran were also sequenced for cyt*b*. The phylogenetic tree revealed that samples of individuals from Northern Iran, Southern Iran and the Toulouse lab aggregate in the same clade. Results of K2P showed that the pairwise genetic distance of *O. rufescens* was closer to that of *Conothoa* subgenus than to others. It seems that the phylogenetic status of *O. rufescens*, is more related to the *Conothoa* subgenera than to *Ochotona* or *Pika*.

Keywords: *Ochotona rufescens*, taxonomic position, cyt*b*, mtDNA and Pika.

Introduction

The phylogeny of the genus *Ochotona* is somewhat confusing (Yu *et al.* 2000) and in the Palearctic region is not completely resolved (Fostowicz-Frelik *et al.* 2010). Based on previous studies, species of the genus *Ochotona* exhibited higher intraspecific differentiation than interspecific variation (Corbet 1978, Smith *et al.* 1990, Erbajeva 1994, Lanier and Olson 2009). These results suggested that a single sample (Tissue samples of populations) is not sufficient to determine the phylogenetic status of the species (Peters *et al.* 2005). Therefore, phylogeny of the genus *Ochotona* must be resolved with more specimen (Hoffmann and

Smith 2005, Lanier and Olson 2009). Although several studies have been performed on Asian Pikas based on the fossil record and molecular phylogenetic relationships (Ning and Changlin, 1996, Niu *et al.* 2004, Cermak *et al.* 2006, Yu *et al.* 2009), the phylogenetic relationship of the Afghan Pika (*O. rufescens*) have been neglected. There are several morphological and molecular studies on the phylogeny of the genus *Ochotona* that used *O. rufescens* for their analysis. However, there have been no molecular genetic studies directly focusing on the phylogenetic status of the Afghan Pika.

Lyon (1904) divided the genus *Ochotona* into three subgenera: *Ochotona*, *Conothoa and Pika* using a morphological study (the names were updated by Fostowicz-Frelik *et al.* 2010). He revealed that *O. rufescens* did not include in the three main subgenera. Erbajeva (1988) based on morphological study, showed that the Afghan Pika belonged to the subgenus *Ochotona*. Niu *et al.* (2004) suggested that the Afghan Pika related to the surrounding Qinghai-Tibet plateau group based on partial cytochrome *b* (cyt*b*) gene (402 bp). They showed based on maximum parsimony that the Afghan Pika is a sister group to *O. roylei himalayana* (*O. himalayana*) and also to *O. forresti* using a neighbour-joining tree with low support (<50 bootstrap). They also showed this species was a sister group of *O. forresti* based on maximum likelihood with medium support (bootstrap=59).

The taxonomic review, based on previous publications (Mainly on Yu *et al.* 2000), Hoffmann and Smith (2005) showed that *O. rufescens* was related to the subgenus *Ochotona,* while Fostowicz-Frelik *et al.* (2010) revealed that the Afghan Pika belongs to the *Pika* subgenera and is a sister group of *O. dodogolica*. Other studies performed by Lanier and Olson (2009), using complete cyt*b* and the ND4 region of mtDNA, and Lissovsky (2014), using complete cyt*b* region of mtDNA, found that this species belonged to the *Conothoa* subgenus and also found that *O. roylei himalayana (O. himalayana), O. roylei, O. ladacensis, O. koslowi*, *O. macrotis* and *O. rutilla* were the sister groups of *O. rufescens* with high support (>60 bootstrap). Totally, one line of evidence suggested that the study species belongs to the subgenus Ochotona, the second to the Pika, while the third body of evidence suggested belonging to Conothoa. In addition, the first set of studies suggested both *O. roylei himalayana* (*O. himalayana*) and *O. forresti* as sister species depending from the criterion and methodological procedure employed for resolving the phylogenetic tree.

Yet, the second set of studies suggested *O. dodogolica* as to be the sister group of the Afghan species, while the third set of studies suggested that it was part of a large clade comprising several species like O. *roylei himalayana (O. himalayana), O. roylei, O. ladacensis*, *O. koslowi*, *O. macrotis* and *O. rutilla.* To provide adequate phylogenetic resolution for the genus *Ochotona*, using more species and intraspecific samples could be important (Lanier and Olson 2009). However, up to now all studies on the phylogenetic relationship between the Afghan Pika and other Pikas have been performed with only one or two samples of Afghan Pika.

The controversial systematics of the Afghan pika, and of the whole group, could indeed arise for the limited use of just one or two specimens of this taxon. According to Lanier and Olson (2009) it is necessary to use more species and intraspecific samples to provide an adequate phylogenetic resolution for the genus *Ochotona*. In agreement with these authors we therefore employed more than 20 specimens from different sample locations, with the aim of testing whether this enhancement could help to reassess the phylogenetic relationships of the Afghan Pika.

Among the 13 types of mitochondrial DNA protein coding genes we selected the cyt*b* both

because for its performances in discovering the true phylogenetic relationships among species (Zardoya and Meyer 1996, Yu *et al.* 2000), but also because for comparative reasons as this genes was aleready employed in former studies on the Afghan pika (Irwin *et al.* 1991, Yu *et al.* 2000).

Material and methods

We retrieved sequences of cyt*b* gene (1140 bp) of various pika species from GenBank. Sequences for Afghan Pika were newly obtained from 25 specimens that were trapped in the North Khorasan province (Iran) between August and November 2012 (Fig. 1). Two cyt*b* sequences for the Afghan Pika were found in GenBank. These sequences also were used in our analysis. The whole genomic DNA was extracted from preserved (96% ethanol) tissues (liver and muscle) using AccuPrep genomic DNA Extraction Tissue Kit (Bioneer) following the manufacturer's instructions. The PCR mix (AccuPower® PCR premix Kit, Bioneer) in the volume of 25 µl included 1 U of *Top* DNA polymerase, 10 µM Tris-HCl, 30 µM KCl, 1.5 µM $MgCl_2$, and 250 µM each of dNTP and 2 pmol primer. The cyt*b* mitochondrial genes were amplified using the primers H15915 (TGCTCTCCTTCTCTGGTTTACAAGAC) and L1472 (TGACTAATGATATGAAAAAC CATCGTTG), as described in Lerp *et al.* (2011).

An Applied Biosystems thermal cycler (version 2.09) was used for PCR amplification. The PCR protocol for tissue samples consisted of an initial 180 sec denaturation step at 95 °C, five cycles of denaturation at 94 °C for 60 sec, primer annealing at 45 °C for 90 sec and extension at 72 °C for 90 sec, then 40 cycle steps of 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C, and a final 10 min extension step at 72 °C. The Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) was used for double-strand cycle sequencing according to the manufacturer's instructions, and the ABI PRISM 3730xl automatic sequencer was used for electrophoresis of the purified sequencing product. Sequences were devised and edited with Seqscape 2.6 (Applied Biosystems). Mega 6 (Tamura *et al.* 2013) was used for the alignment of sequences with the ClustalW method. The cyt *b* sequences were deposited in GenBank under accession numbers KJ958163-KJ958194. The Kimura two-parameter (K2P) distances were calculated using Arlequin 3.5 (Excoffier and Lischer 2010). For reconstruction of the phylogenetic tree, different cyt*b* sequences of the genus *Ochotona* were retrieved from GenBank and aligned manually with the *O. rufescens* sequences from Iran. We used *Lepus europaeus* and *Oryctolagus cuniculus* as outgroups (Lanier and Olson 2009).

The phylogenetic tree was inferred from maximum likelihood (ML) and Bayesian Inference (BI). We used the JModelTest 2.1.3 (Posada 2008) to determine which model of evolution best fitted the data. Analysis showed that the GTR model with gamma shape parameter (G = 1.09) and proportion of invariable positions (I = 0.51) is the best fitted model. The ML tree was performed with PAUP 4.0 (Swofford 2002) with heuristic searches. We constructed the BI tree in MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003). Analysis was performed with Markov chain Monte Carlo sampling for 10 million generations and sampled every 100 generations. Only values >50% were considered. The first 25% of trees were discarded as burn-in.

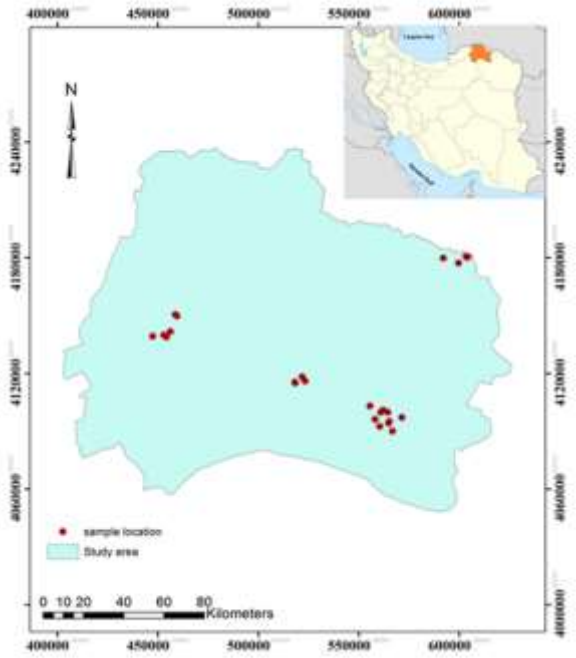
Results

In total, 355 different sequences related to different Pika species were retrieved from GenBank and 25 individuals from North Khorasan province, Iran, were sequenced for a cyt*b* mitochondrial gene. The final data set with 1140 bp was studied for each individual. ML and BI showed almost the same topology

of phylogenetic trees, but the BI tree showed higher posterior probabilities among species. The results confirmed that there are five distinct subgenera in the genus *Ochotona*. Three common subgenera (*Ochotona*, *Pika* and *Conothoa*) were recognized and *O. pusilla* and *O. syrinx (O. huangensis)* were separated from other species of Pika (Fig. 2). In the *Conothoa* subgenera, three groups were recognized based on both maximum likelihood and Bayesian analysis trees (Fig. 2) in the BI and ML trees, our focal taxon, *O. rufescens* was separated from other species of subgenera.

Within *O. rufescens*, two groups could be separated. Group 1 included specimen from both Northern Iran and the Toulouse laboratory specimens, while group 2 also included the Southern Iran (Kerman province) specimens. *O. erythrotis* and *O. forresti* were sister groups and were placed in another group. *O. roylei himalayana (O. himalayana), O. roylei*, *O. macrotis*, *O. ladacensis*, *O. rutila and O. koslowi* were placed in a different group.

Figure 1. Geographical distribution of the sampling sites for Afghan Pika in Iran.



The results of K2P showed that the pairwise genetic distance between our samples and those of the GenBank specimens of Afghan Pika is not higher than 10%. The nearest species based on pairwise genetic distance (Kimura two-parameter) to *O. rufescens* were *O. roylei*, *O. roylei himalayana (O. himalayana)* and *O. forresti*. Overall, the pairwise genetic distance of *O. rufescens* was closer to species of the *Conothoa* subgenus than to others (Table 1).

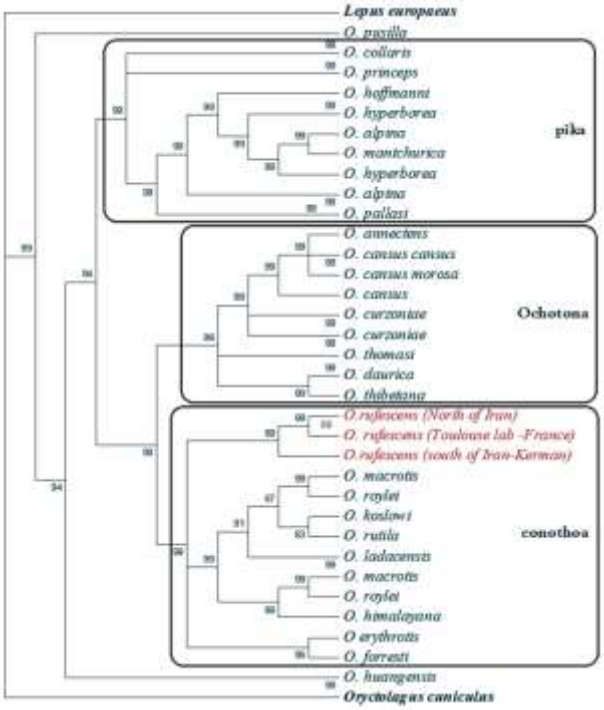


Figure 2. Phylogenetic relationship between species of pika (genus *Ochotona)* based on Bayesian inference using MrBayes. Nodal support indicated by posterior probabilities × 100 on top of the branch.

Discussion

Based on phylogenetic cyt*b* analysis, the monophyly of the main subgenera of genus *Ochotona* (*Conothoa*, *Pika* and *Ochotona*) described by Lyon (1904), Yu *et al*. (2000), Lanier and Olson (2009) and Lissovsky (2014) is supported with high value Bayesian posterior probabilities × 100 (*Conothoa* = 99, *Ochotona* = 98 and *Pika* = 99). The rufescens Iranian clade was a sister group of a much larger group comprising at least two different clades. The

first is formed by *O. roylei himalyana (O. himalyana), O. macrotis, O. roylei, O. ladacensis, O. rutila, O. koslowi* and the second clade groups together *O. forresti* and *O. erythrotis.* All these species belonged to the large subgenus *Conothoa*. Contrariwise to Hoffmann and Smith (2005) who placed *O. rufescens* in the *Ochotona* clade, our analysis confirmed its placement within the *Conothoa*

clade, as suggested by Lanier and Olson (2009) and Lissovsky (2014). The phylogenetic tree revealed that samples from individuals of Northern Iran, Southern Iran and the Toulouse laboratory group into same clade but in different branches. Result of K2P not showed more than 10% pairwise genetic distance between our samples and the GenBank samples of Afghan Pika (5.3% between our samples and the Kerman sample and 3.9% between our samples and the Toulouse samples). According to the standard divergence within a species which may commonly occur under 10% (Billington and Hebert, 1991) and 9.7% based on overall mean distance of all individuals in Mega 6. It seems therefore that, based on our current phylogenetic analysis and K2P distance, there could not be separation among the three Iranian locations so far investigated. However, for a definite assessment of intraspecific variation within the Iranian *O. rufescens*, more samples should be collected from Kerman and other areas of Iran. The molecular phylogenetic survey of *Ochotona* genus by Niu *et al.* (2004) showed that *O. rufescens* has just one sister group based on maximum parsimony, *O. roylei himalayana (O. himalayana)*; one with neighbour joining with low support (<50 bootstrap), *O. forresti* and one with maximum likelihood with medium support (bootstrap=59), *O. forresti*. The sister taxa pair between *O. rufescens* and *O. forresti* or *O. roylei himalayana (O. himalayana)* (Niu *et al.* 2004) is not supported by our study. Since the results of the larger fragments of DNA are more reliable than the smaller fragments (Lanier and Olson 2009), it seems that the short sequences applied by Niu *et al.* (2004) caused unreliable results in their study. Our results repeated the results of Lanier and Olson (2009) and

Table 1. Kimura 2 parameter distance between *O. rufescens* and 23 spieces of *ochotona* genus. Five major subgenus separated from each other. *O. rufescens* (north) = sampled individuals from North Khorasan (north of Iran), *O. rufescens* (Kerman, south of Iran) = one specimen from Genbank and related to Kerman province (South of Iran) and *O. rufescens* (TL) = Toulouse laboratory (France) sample.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1- *O. rufescens* (North)	0																											
2- *O. rufescens* (Kerman)	0.05	0																										
3- *O. rufescens* (TL)	0.04	0.04	0																									
4- *O. himalyana*	0.11	0.11	0.12	0																								
5- *O. roylei*	0.11	0.11	0.12	0.07	0																							
6- *O. forresti*	0.11	0.12	0.12	0.12	0.12	0																						
7- *O. macrotis*	0.12	0.12	0.13	0.08	0.07	0.12	0																					
8- *O. erythrotis*	0.13	0.12	0.13	0.13	0.12	0.12	0.13	0																				
9- *O. curzoniae*	0.13	0.13	0.14	0.13	0.12	0.14	0.12	0.14	0																			
10- *O. cansus*	0.14	0.13	0.14	0.13	0.13	0.13	0.14	0.15	0.15	0																		
11- *O. princeps*	0.14	0.14	0.15	0.14	0.14	0.14	0.15	0.14	0.14	0.09	0																	
12- *O. hoffmanni*	0.14	0.15	0.15	0.13	0.13	0.14	0.13	0.15	0.14	0.15	0.17	0																
13- *O. annectens*	0.16	0.15	0.16	0.15	0.15	0.13	0.15	0.16	0.16	0.13	0.14	0.17	0															
14- *O. cansus*	0.15	0.16	0.16	0.15	0.15	0.14	0.15	0.16	0.16	0.13	0.15	0.17	0.02	0														
15- *O. curzoniae*	0.16	0.16	0.16	0.15	0.15	0.15	0.16	0.16	0.16	0.14	0.14	0.16	0.06	0.07	0													
16- *O. dauurica*	0.16	0.17	0.18	0.17	0.16	0.15	0.17	0.17	0.17	0.16	0.16	0.18	0.09	0.09	0.1	0												
17- *O. pallasi*	0.16	0.16	0.17	0.15	0.16	0.16	0.16	0.14	0.16	0.12	0.12	0.16	0.16	0.16	0.16	0.16	0											
18- *O. huangensis*	0.16	0.15	0.17	0.15	0.15	0.16	0.16	0.15	0.17	0.14	0.14	0.16	0.15	0.14	0.14	0.16	0.15	0										
19- *O. hyperborea*	0.16	0.16	0.16	0.14	0.15	0.15	0.16	0.15	0.15	0.11	0.1	0.17	0.14	0.15	0.15	0.16	0.11	0.15	0									
20- *O. koslowi*	0.13	0.13	0.13	0.12	0.11	0.13	0.13	0.14	0.11	0.16	0.15	0.13	0.15	0.16	0.15	0.17	0.15	0.17	0.15	0								
21- *O. nubrica*	0.15	0.16	0.16	0.15	0.15	0.15	0.15	0.16	0.16	0.14	0.14	0.16	0.06	0.07	0.02	0.1	0.16	0.14	0.15	0.15	0							
22- *O. thomasi*	0.15	0.16	0.16	0.15	0.14	0.15	0.15	0.16	0.16	0.15	0.13	0.16	0.09	0.09	0.09	0.1	0.15	0.15	0.15	0.16	0.09	0						
23- *O. thibetana*	0.16	0.17	0.17	0.16	0.16	0.15	0.16	0.17	0.17	0.15	0.15	0.18	0.09	0.09	0.09	0.1	0.16	0.16	0.15	0.17	0.09	0.1	0					
24- *O. hoffmanni*	0.16	0.17	0.17	0.16	0.16	0.15	0.17	0.15	0.16	0.13	0.12	0.17	0.15	0.16	0.15	0.16	0.11	0.16	0.09	0.16	0.15	0.14	0.15	0				
25- *O. alpina*	0.16	0.17	0.17	0.16	0.16	0.15	0.17	0.16	0.16	0.13	0.12	0.17	0.15	0.16	0.16	0.17	0.11	0.16	0.09	0.17	0.16	0.15	0.15	0.06	0			
26- *O. dauurica*	0.16	0.16	0.17	0.18	0.17	0.18	0.17	0.16	0.17	0.16	0.16	0.18	0.09	0.09	0.1	0.02	0.16	0.17	0.16	0.16	0.1	0.1	0.1	0.16	0.16	0		
27- *O. pusilla*	0.2	0.18	0.2	0.19	0.19	0.19	0.2	0.18	0.19	0.16	0.17	0.19	0.18	0.19	0.17	0.18	0.16	0.17	0.17	0.19	0.17	0.17	0.17	0.17	0.18	0.19	0	
28- *O. mantchurica*	0.17	0.17	0.18	0.16	0.17	0.16	0.17	0.16	0.17	0.13	0.12	0.18	0.18	0.18	0.16	0.18	0.12	0.16	0.06	0.17	0.15	0.16	0.15	0.09	0.08	0.18	0.17	0

Lissovsky (2014). The *O. macrotis*, *O. roylei*, *O. ladacensis*, *O. koslowi* and *O. iliensis* were sister groups of *O. rufescens* (>50 bootstrap). It seems that, based on the K2P distances between *O. rufescens* and species of the genera *Ochotona* (Table 1), and the resulting phylogenetic trees, the phylogenetic position of *O. rufescens* is more related to the *Conothoa* subgenera than to *Ochotona* or *Pika*.

Acknowledgment

We thanks to anonymous reviewers provided helpful comments on earlier drafts.

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