



## Short Communication

## Genetics, morphology and ecology reveal a cryptic pika lineage in the Sikkim Himalaya



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## ABSTRACT

Asian pika species are morphologically ~similar and have overlapping ranges. This leads to uncertainty and species misidentification in the field. Phylogenetic analyses of such misidentified samples leads to taxonomic ambiguity. The ecology of many pika species remains understudied, particularly in the Himalaya, where sympatric species could be separated by elevation and/or substrate. We sampled, measured, and acquired genetic data from pikas in the Sikkim Himalaya. Our analyses revealed a cryptic lineage, *Ochotona sikimaria*, previously reported as a subspecies of *O. thibetana*. The results support the elevation of this lineage to the species level, as it is genetically divergent from *O. thibetana*, as well as sister species, *O. cansus* (endemic to central China) and *O. curzoniae* (endemic to the Tibetan plateau). The Sikkim lineage diverged from its sister species' about 1.7–0.8 myr ago, coincident with uplift events in the Himalaya. Our results add to the recent spate of cryptic diversity identified from the eastern Himalaya and highlight the need for further study within the Ochotonidae.

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## 1. Introduction

Much of the Earth's biodiversity is restricted to a handful of global biodiversity hotspots. The Eastern Himalaya in Asia is one such hotspot where new species of birds, mammals, and reptiles are continuously discovered (Athreya, 2006; Chakraborty et al., 2007; Agarwal and Karanth, 2015). The Sikkim Himalaya is part of the Eastern Himalaya and is particularly rich in biodiversity (Myers et al., 2000). These mountains have complex topographical features encompassing elevations between 300 m and 8500 m. Globally, mountainous regions tend to have high species richness, partly due to spatial heterogeneity, altitudinal gradients and/or varied climatic conditions. The geographic isolation of high elevation species in combination with topographic complexity creates opportunities for increased genetic differentiation and speciation (Kreft and Jetz, 2007; Robin et al., 2015). Montane small mammals may even show higher levels of endemism and increased rates of genetic differentiation due to their limited dispersal ability. The

extent of genetic differentiation of biota remains largely unexplored in much of the Himalaya owing to inadequate systematic sampling efforts that can obscure realistic estimates of diversity in this mountainous region.

The genus *Ochotona* Link, 1795 (Family Ochotonidae), commonly known as pikas, represents one of the most common cold-adapted herbivores occurring in the higher altitudes of the Himalaya. The genus is largely Asian, with 26 of the 28 known species reported from this part of the world (Lisovsky, 2014). Because pikas are uniquely adapted to cold environments and/or high-elevation regions, they are an exemplary group to investigate endemism and diversity. Five species (*Ochotona curzoniae*, *O. roylei*, *O. macrotis*, *O. forresti* and *O. thibetana*) have been reported from the Eastern Himalaya, including the Sikkim Himalaya (Hoffmann and Smith, 2005). All Eastern Himalayan species belong either to the subgenus *Ochotona* ('shrub-steppe' group) or *Conothoa* ('mountain' group), both characterized by a complex taxonomy.

Occurring in remote high elevations of the Himalaya, pika species remain poorly studied. This may be the case for *O. thibetana sikimaria* Thomas, 1922, which has a disjunct distribution from other subspecies of *O. thibetana* Milne-Edwards, 1871. We explored the possibility of cryptic diversity in *O. thibetana* from the Eastern

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Himalaya. Initially placed within *O. cansus* (Feng and Kao, 1974; Feng and Zheng, 1985), but more recently assigned to *O. thibetana* (based on morphological similarity: Smith et al., 1990), the taxonomy of *O. t. sikimaria* remains uncertain. We used genetic markers (mitochondrial and nuclear DNA), and skull morphometrics to analyze the taxonomic position of *O. t. sikimaria*. We sampled all putative sister species and investigated (1) whether *O. t. sikimaria* represents a distinct phylogenetic unit when compared to *O. thibetana* and (2) the evolutionary history and placement of *O. t. sikimaria* in the pika phylogeny. Our results suggest recognition of this clade as a distinct species, and we hereafter refer to it as *O. sikimaria*.

## 2. Materials and methods

### 2.1. Sampling

We investigated the phylogenetic placement of *O. sikimaria*, by also sampling three related species (*O. curzoniae*, *O. thibetana*, and *O. cansus*) (Appendix 1). Potential pika habitats in Sikkim, Tawang districts of Arunachal Pradesh (Eastern Himalayan states of India), Central Nepal (Annapurna Conservation Area) and Langtang National Park (Nepal) were sampled using fecal pellets as cue and also direct sightings during field visits between May 2011 and April 2013. Live-trapping was conducted in these habitats, with each location sampled. Sherman live traps and Tomahawk traps baited with fresh vegetation, apples, and carrots were used (20–50 traps per site for at least 3 days). Tissue samples were collected using an ear punch. Animals were released after handling. Basic ecological data were also recorded during sampling (Appendix 2).

### 2.2. Craniometric analysis

A subset of pika species were used for craniometrical analysis. Collections of pikas from the ‘thibetana’ group: *O. thibetana*, *O. sikimaria*, *O. cansus* and *O. syrinx* from different museums were included. In order to avoid inconsistencies, all measurements were made by A. Lisovsky. As a result, we were unable to include measurements for some of the individuals for which we have genetic samples. Three specimens of *O. cansus* from the same locality (Maerkang) from the Beijing Institute of Zoology were included, as were *O. thibetana* and *O. syrinx* specimens (Appendix 3). All morphological and genetic sampling are shown in Appendices 3 and 4. We did not include specimens of *O. curzoniae* in our analyses because extensive morphological differentiation between *O. curzoniae* and species from the ‘thibetana’ group has been established (Lisovsky, 2014), and including *O. curzoniae* could mask differences between the more similar species within the ‘thibetana’ group.

We took 20 measurements (following Lisovsky, 2014) of 188 intact skulls (Appendix 3). All calculations were carried out on Log 10-transformed measurements. Data on sex, collection date, collector, and body measurements were taken from the museum labels and incorporated into a database. Each specimen was assigned to one of three age groups: (1) juveniles with undeveloped crests on the mandible and an arc-shaped profile of the skull, together with shortened nasals, (2) subadults with a fully developed skull profile, but with undeveloped crests on the mandible, or (3) adults (Lisovsky, 2004). Pikas have no sexual dimorphism in skull shape (Lisovsky, 2014), and thus, we did not separate males and females. To exclude age bias, an orthogonal projection of initial data along the vector of age variation (Burnaby, 1966) was used. The vector of age variation was calculated as the first eigenvector of the between-group covariance matrix computed with a nested two-factor MANOVA, in which the variable contain-

ing three age gradations, as well as the identifier of the one-species geographic samples, were used as grouping variables. The age factor was nested within the geographic samples. We used only the classes 1 and 3 to calculate the covariance matrix, in order to minimize errors arising from inaccuracy in determining age class 2 (Lisovsky, 2014). We used three geographical samples across two age classes to calculate the vector of age variation.

Ordination was conducted following Lisovsky (2014). In summary, the initial data were rotated into the space of intergroup variation without distortion of the initial space. Standard modules of Statistica, version 8 (Statsoft Inc., Tulsa, USA), as well as algorithms, written by A. Lisovsky in Statistica Visual Basic, were used in the craniometric analyses.

### 2.3. DNA isolation, amplification and sequencing

Total genomic DNA was isolated from tissue samples using commercially available DNAeasy Blood and tissue kit (Qiagen Inc.) following the manufacturer's instructions. Sequences of three mitochondrial loci: cytochrome *b* (1140 bp), ND4 (658–1300 bp), d-loop (450–635 bp); as well as partial Rag1 (500–735 bp) and partial Rag2 (464–507 bp) were attempted for 19 samples (see Appendix 4, Table 2). A standard PCR protocol was used to amplify the selected genes. Supplementary Table 1 includes details of primers and annealing temperatures. PCR products were visualized on a 2% Agarose gel and sequenced on an automated sequencer 3730 Genetic Analyzer (Applied Biosystems, Thermo Scientific, USA) in both directions. De novo assembly was performed in Geneious 6.0.3 (Biomatters) after checking for insertions, deletions, and stop codons. Twelve out of 28 samples yielded at least two independently evolving genes and were used for species tree analysis (see Appendix 4, Table 2).

### 2.4. Phylogeny, species delimitation, divergence estimation and genetic distance

We used three datasets to generate trees – (1) cytochrome *b* (Appendix 4); (2) concatenated mitochondrial (cytochrome *b* and ND4) for divergence estimate (Appendix 4, Table 1); and (3) cytochrome *b*, ND 4, Rag 1 and Rag2 to generate a coalescent-based Bayesian multilocus species tree (Appendix 4, Table 2; Fig. 2b). Alignment and .xml files are deposited in Dryad, (doi: <http://dx.doi.org/10.5061/dryad.24jk4>). The substitution model of best fit for each gene was assessed using Partition finder V1.1.1 (Lanfear et al., 2012).

Cytochrome *b* tree: Bayesian posteriors and topology were estimated using MrBayes (3.2.1) (Ronquist and Huelsenbeck, 2003). Analyses were run using five chains in parallel and were stopped when the standard deviation of split frequency was <0.01 and the potential scale reduction factor (PSRF) of each parameter was equal to 1. The trace file was also examined in Tracer v1.6 (Rambaut et al., 2014) to examine convergence. A maximum likelihood (ML) tree was computed in RAxML GUI v1.3 (Silvestro and Michalak, 2012) to check for congruency of the topology. We used the general time-reversal model (GTR) on codon-partitioned (1 + 2 + 3) datasets. Fourteen species of rabbits and hares were used as outgroups (Appendix 4).

Species tree: We used a coalescent-based Bayesian species tree method implemented in \*BEAST (Heled and Drummond, 2010) on a multilocus dataset with a subset of candidate species (8 taxa belonging to the subgenus *Ochotona*). Three species of the subgenus *Pika* were selected as outgroups. The input files were created using BEAUti, and the partition schemes suggested by Partition finder V1.1.1 (Lanfear et al., 2012) were used; cytochrome *b*, ND4 and Rag 1 sequences were partitioned by three codon positions and Rag 2 was partitioned by two codon partitions (1 + 2) + 3. An unlinked

substitution model was used for partition and speciation and Yule process was used as a tree prior.

Convergence of the run was checked using TRACERv1.6 (Rambaut et al., 2014) and the runs were combined using the Log combiner module of BEAST with burnin of 10%. Fig tree v1.4 (Rambaut, 2012) was used to visualize the trees.

Species delimitation: RAXML gene trees were used in Bayesian Poisson Tree Processes (bPTP), as implemented in bPTP server (<http://species.h-its.org/ptp/>; Zhang et al., 2013). Species delimitation analyses were conducted for cytochrome *b* and ND4 sequences as these regions have the best taxon sampling for pikas. Additionally, GMYC (Generalized Mixed Yule Coalescent) species delimitation tool (Pons et al., 2006) was tested on the ultrametric BEAST tree, using the SPLITS package (available from <http://r-forge.r-project.org/projects/splits/>) implemented in R software.

Genetic distance: The codon partitioned model determined by Treefinder (Jobb, 2011) was used to calculate between-group pairwise genetic distances for the cytochrome *b* gene.

Divergence estimate: Divergence of *O. sikimaria* was estimated using a lognormal relaxed molecular clock (uncorrelated) method, implemented in the program BEAST v1.8.1 (Drummond et al., 2012). Concatenated sequences of mitochondrial origins – cytochrome *b* and ND4, were used for the analyses. The Leporidae–Ochotonidae divergence of 31 myr (as in Lanier and Olson, 2009) was used as a calibration point. Because divergence estimation failed to reach convergence (ESS < 200) even after 800 million generations for certain parameters, a simpler model, TN93 + G + I, was applied to partitions than the suggested GTR + G + I model. We enforced the Bayesian tree topology for divergence dating.

### 3. Results

#### 3.1. Morphology

Individuals of *O. sikimaria* fall within the cranial variation of *O. thibetana* (Supplementary Fig. 1). The difference between these two species is the size of their auditory bullae and distance between them (with larger bullae in *O. thibetana*). However, all measurements overlap. Specimens of *O. sikimaria* and *O. cansus* are distinct, and differ mostly in skull width (6 measurements along the skull axis), with a wider skull in *O. sikimaria*.

One of our genetic samples had skull measurements within the morphospace of *O. sikimaria*. This sample is situated at the edge of the *O. sikimaria* cranial distribution, however not far from the type specimen of *O. sikimaria*.

#### 3.2. Distribution range and ecology of *O. sikimaria*

A detailed table comparing habitats of all closely related species of *O. sikimaria* is given in Appendix 2. We recorded *O. sikimaria* from different localities in the Sikkim Himalaya starting from elevations of 2600 m, which corresponds to mixed conifer forest, to 4754 m which is regarded sub-alpine habitat. Fecal pellet surveys in Central Nepal and Tawang district of Arunachal Pradesh followed by genetic typing did not reveal the presence of *O. sikimaria* from these locations.

The distribution range of *O. sikimaria* is disjunct from *O. cansus* and *O. thibetana*, separated by at least 1200 km (aerial distance). While *O. sikimaria* and *O. curzoniae* are found in relatively close

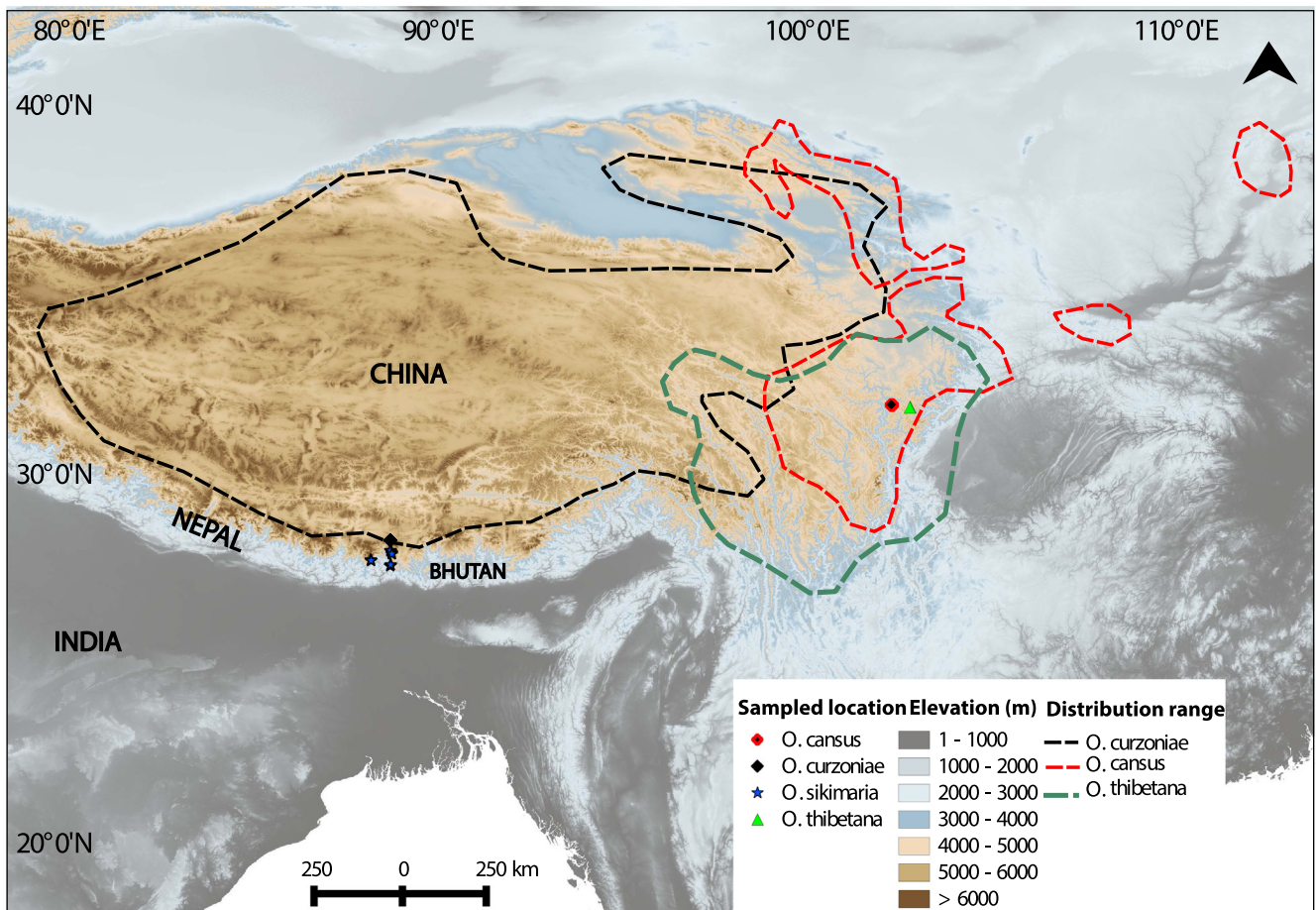


Fig. 1. Sampling locations and species distribution ranges plotted on an elevation map (Worldclim).

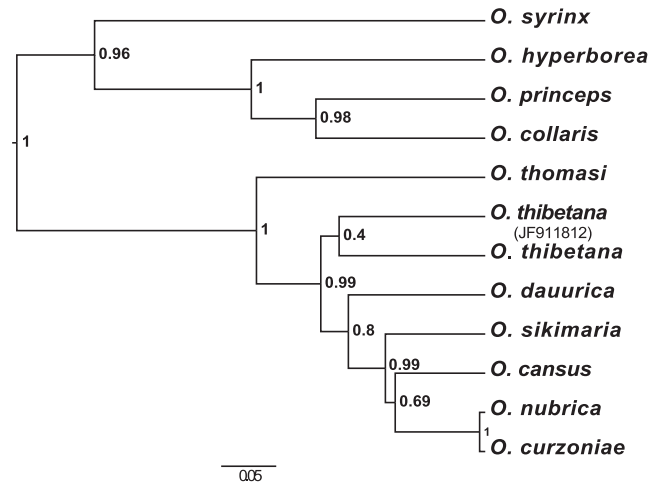


proximity (separated approximately by 30 km aerial distance), they inhabit very distinct habitats (Appendix 2). The burrowing sites of *O. sikimaria* are generally under permanent rocks. They also use fallen logs. They feed on a variety of vegetation, including bark of apple trees, mosses, *Rhododendron* spp. flowers, as well as yak dung. Populations near human settlements opportunistically feed on young saplings of cabbage, peas, and potatoes.

### 3.3. Phylogeny, species delimitation, divergence estimate and genetic distance

*Ochotona sikimaria* is monophyletic in the mitochondrial tree. Although the cytochrome *b* gene sequences are available for most species of pikas, the tree generated (Fig. 2a) failed to resolve the relationships between *O. sikimaria*, *O. cansus* and *O. curzoniae*. These three species form an unresolved trichotomy. *Ochotona thibetana* (JF911812) from Sichuan, Shimian County, renders *O. thibetana* paraphyletic, like in a previous study (Lissovsky, 2014). Our samples of *O. thibetana* from Li County, Sichuan, China form a monophyletic clade with *O. thibetana* (AF272986) and *O. thibetana nangqenica* (JF911813) from Qinghai (Fig. 2a).

A multi-locus species tree (Fig. 2b) was able to resolve the position of *O. sikimaria* within the *Ochotona* subgenus, sister to



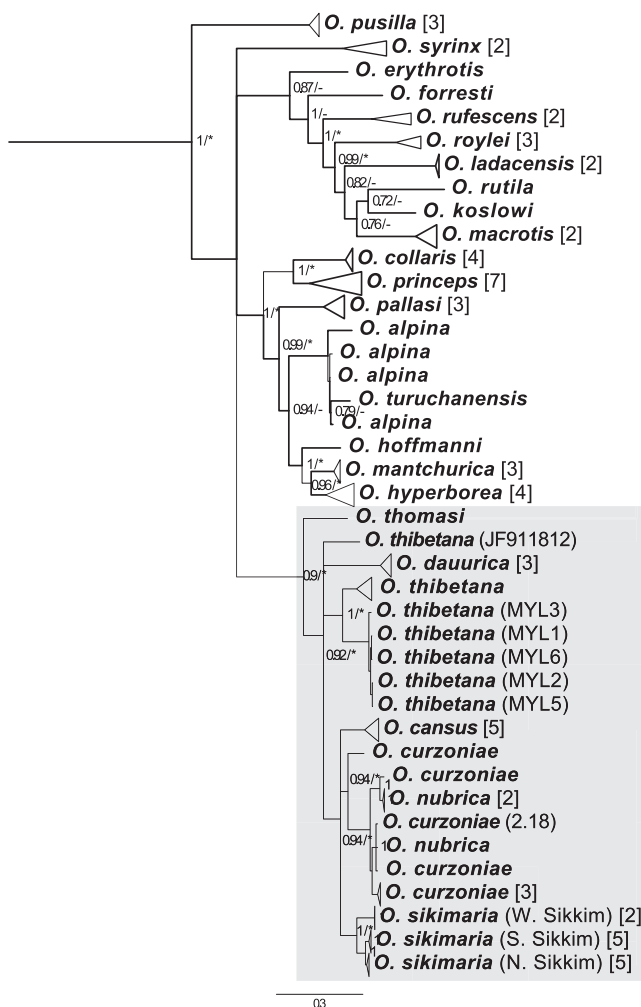
**Fig. 2b.** Bayesian inference of Species Trees (\*BEAST) of a subset of candidate pika species within the sub-genus *Ochotona*. Three species of the sub-genus *Pika* were selected as an out group. Nodal support indicated by posterior probabilities is shown.

*O. cansus* and *O. curzoniae*. *Ochotona sikimaria* samples from West Sikkim appeared distinct from the rest of the samples in the mitochondrial tree (Fig. 2a). *Ochotona thibetana* was an outgroup to the three species complex (*O. cansus*, *O. curzoniae* and *O. sikimaria*), distinct from *O. sikimaria* (Fig. 2b). Mitochondrial species delimitation results for *O. sikimaria* are summarized in Supplementary Table 2. Both GMYC and bPTP, for ND4 and cytochrome *b* genes consistently confirmed *O. sikimaria* as a distinct species. Additional putative cryptic lineages are described in Supplementary Table 2.

We estimated the divergence of *O. sikimaria* from its sister species to be 1.3 myr (95% HPD 1.7–0.8) (Supplementary Table 3). The interspecies genetic distances between *O. sikimaria* and its closest relatives are shown in Supplementary Table 4. *Ochotona sikimaria* is closest to *O. cansus* (genetic distance:  $6.7 \pm 0.04\%$ ); followed by *O. curzoniae* ( $9 \pm 0.05\%$ ) and *O. nubrica* ( $10 \pm 0.04\%$ ). The distance between *O. sikimaria* and *O. thibetana* was  $12 \pm 0.04\%$ .

## 4. Discussion

We explored the taxonomic position of one of the most common small mammals in the Sikkim Himalaya using morphological, genetic, and ecological data. Phylogenetic analyses place *O. sikimaria* as a distinct, coherent clade, sister to *O. cansus* and *O. curzoniae*. The estimated species tree based on the multilocus dataset, the mitochondrial gene tree, and the morphometric analyses clarifies that *O. sikimaria* is not a taxon of sub-specific rank within *O. thibetana* and/or *O. cansus* as hypothesized by previous authors (Feng and Zheng, 1985; Smith et al., 1990). We are convinced of the taxonomic distinctiveness of *O. sikimaria* because (a) there is a low morphological difference between our genetically sampled individuals and the *O. sikimaria* museum collections (b) we genetically sampled individuals from the same locations as museum specimens previously identified as *O. sikimaria*. While distinct genetically, *O. sikimaria* is similar to *O. thibetana* morphologically. The genetic distance between *O. sikimaria* and *O. thibetana*, *O. cansus*, and *O. curzoniae* is comparable to interspecific genetic distances within the subgenus *Ochotona* (Supplementary Table 3). This implies that *O. sikimaria* has been evolving independently and is deeply divergent from *O. thibetana*. Finally, species delimitation analyses suggest *O. sikimaria* is a distinct clade, with possible cryptic geographic variation. Within this clade, we observe high genetic differentiation across valleys in the Sikkim Himalaya.



**Fig. 2a.** Bayesian phylogeny inferred from mitochondrial cytochrome *b* gene, computed in MrBayes. Nodal support indicated by posterior probabilities and significant bootstrap (>70) values of a maximum likelihood tree are represented as \*, non-significant values indicated as -. The numbers in box brackets represent nodes collapsed and the numbers in round brackets represent voucher numbers of the samples added in the present study.

We estimated the divergence of *O. sikimaria* from its sister species, *O. cansus*, to be 1.3 myr (95% HPD 1.7–0.8) (Supplementary Table 3). Divergence estimates for other species pairs in our tree were comparable to those estimated using nuclear markers in Melo-Ferreira et al. (2015). Time to the most recent common ancestor (TMRCA) for *O. sikimaria* coincides with geological uplift (e.g. Kun-Huang movement, 1.6–0.2 myr) and the maximum extent of Xixiabangma glaciers (1.2–0.8 myr). Divergence times of other montane birds and mammals from the eastern edge of the Tibetan plateau are similar (Zhan et al., 2011; Chakraborty et al., 2007; Fan et al., 2011; Yu et al., 2013). We suggest that divergence and phylogeographic differentiation among these closely related groups of pikas may be the result of mountain uplift, followed by range restriction during glacial periods. We expect similar effects on other sympatric mid- to high-elevation species, such as voles, in this region.

The distribution range of *O. cansus*, *O. thibetana*, *O. curzoniae* and *O. sikimaria* suggest that *O. sikimaria* is isolated from its sister species (Fig. 1). While *O. sikimaria* and *O. curzoniae* are genetically and geographically closer, they differ in their habitat preference. *Ochotona curzoniae* inhabits the cold desert across the Tibetan plateau of China, Nepal and India (including Sikkim) while *O. sikimaria* inhabits mesic meadows near the tree line of the Eastern Himalaya. *Ochotona cansus* is endemic to alpine meadows of Central China.

The phylogenetic position of *O. sikimaria* based on mitochondrial and nuclear trees are concordant, suggesting the lack of introgression. However, we find discordance between morphological and genetic data. Interestingly, *O. sikimaria* is genetically deeply divergent and not even sister to the species with which it shares the most morphological similarity. It is possible that this discordance between genetic and morphometric data may be the result of strong stabilizing selection on morphology operating in mountainous terrain (Bickford et al., 2007; Egea et al., 2016). A more detailed study investigating correlations between genetic and morphological diversity across Himalayan species may yield more insights into these processes.

## 5. Conclusion

We identify a reciprocally monophyletic, divergent pika lineage within the Eastern Himalaya. We propose this lineage be recognized as a separate species called *O. sikimaria*. This species is the most common pika in relatively lower elevations of the Sikkim Himalaya. Thought to be a subspecies of *O. thibetana*, *O. sikimaria* is sister to *O. cansus* (from central China) and *O. curzoniae* (from the Tibetan plateau). The earlier misidentification of *O. sikimaria* as *O. thibetana* was due to their morphological similarity.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2016.09.015>.

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