The phylogeny of pikas (*Ochotona*) inferred from a multilocus coalescent approach

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Abstract

The clarification of the systematics of pikas (genus *Ochotona*) has been hindered by largely overlapping morphological characters among species and the lack of a comprehensive molecular phylogeny. Here we estimate the first multilocus phylogeny of the genus to date, by analysing 12 nuclear DNA markers (total of 7.5 Kb) in 11 species of pikas from the four classified subgenera (*Pika*, *Ochotona*, *Lagotona* and *Conothoa*) using a multispecies coalescent-based framework. The species-tree confirmed the subgeneric classification by retrieving as monophyletic the subgenera represented here by more than one species. Contrary to previous phylogenies based on mtDNA alone, *Lagotona* was found to be sister to *Pika*. Also, support for the monophyly of the alpina group was not strong, thus caution should be used in future analyses of this group. A relaxed molecular clock calibrated using the Ochotonidae-Leporidae divergence resulted in more recent estimates of divergence times relative to previous studies. Strong concordance with inferences based on fossil records was found, suggesting that the initial diversification of the genus took place by the end of late Miocene. Finally, this work sets up methodologies and gathers molecular markers that can be used to extend the understanding of the evolutionary history of the genus.

Keywords: Multilocus Coalescent; *Ochotona*; Pika; Relaxed Molecular Clock; Species-tree; Systematics.

1. Introduction

Methods that allow reconstructing the phylogeny of species in a multilocus perspective, taking into account the coalescence of different loci, provide a good opportunity to clarify the systematics of taxonomic groups with traditionally confusing classifications and evolutionary histories. This

remains true in cases where phylogenies based on the widely used mitochondrial DNA are the sole source of phylogenetic information available at the molecular level, because single-gene phylogenies can often result in erroneous representations of the true species-tree given the variance associated to the evolutionary process among loci (Maddison, 1997). Pikas (family Ochotonidae) are one of such groups.

Pikas comprise a single extant genus, *Ochotona* Link, 1795, and with rabbits and hares (family Leporidae) form the order Lagomorpha. Pikas are endemic to the Holarctic Region and the 28 recognized living species of pikas are currently mostly restricted to Asia (26 species), with the remaining species inhabiting North America (Lissovsky, 2014). However, pikas are known to have had a more extensive distribution range throughout the Pleistocene. For example, even though the steppe pika (*O. pusilla*) is today restricted to the central Russian steppes and northern Kazakhstan, fossil records show that during the Pleistocene its range extended to Western Europe (see e.g. Erbajeva and Zheng, 2005 and references therein).

The attempts to establish a robust phylogeny of pikas have been complicated by the largely overlapping morphological characteristics of extant and fossil species (see Erbajeva and Zheng, 2005; Hoffmann and Smith, 2005) and by the use of limited molecular phylogenetic approaches. Phylogenetic relationships in this group were to date inferred solely based on mitochondrial DNA (Yu et al., 2000; Niu et al., 2004; Lanier and Olson, 2009; Ge et al., 2013; Lissovsky, 2014). Some hypotheses have nevertheless resulted from these studies. For example, Yu et al. (2000) suggested that three major evolutionary groups may exist in *Ochotona*, a northern subgroup, a shrub–steppe dwelling subgroup, and a mountain subgroup, which motivated the partition of species among three subgenera, *Pika*, *Conothoa*, and *Ochotona*, respectively (Hoffmann and Smith, 2005). Later mtDNA phylogenies using a more representative sampling of species suggested some

rearrangements of taxa among subgenera (Lanier and Olson, 2009; Lissovsky, 2014) or even the inclusion of a fourth subgenus, *Lagotona,* comprising only *O. pusilla* (Lissovsky, 2014). However, robust inferences of the relationships among species still await more powerful multilocus analyses.

Here the first multilocus phylogeny to date – 12 nuclear loci for a total of 7.5 kb – was inferred for 11 pika species applying a coalescent-based phylogeny reconstruction method.

2. Materials and methods

2.1. Sampling and laboratory work

A total of 11 pika species, about a third of all presently described *Ochotona* species, and 12 molecular markers were combined in this study. The four subgenera according to Lissovsky (2014), *Conothoa*, *Ochotona*, *Pika*, and *Lagotona*, were represented in the sampling (Table 1; Fig. 1; see ranges of the sampled species in Smith et al., 1990, Lissovsky et al., 2007 and Smith and Xie, 2008), and at least two individuals per species were newly sequenced for each marker. The only exception was *O. princeps* with one newly sequenced individual to which sequences available in GenBank and Ensembl were added to represent a second specimen (see Suppl. Table 1 for accession numbers). Of the analyzed markers, nine were autosomal (ALB, DARC, OXA1L, PPOX, PRKCI, SPTBN1, TSHB, UCP2 and UCP4; Matthee et al., 2004; Alves et al., 2008; Melo-Ferreira et al., 2009; Melo-Ferreira et al., 2012) and three were X-linked (AMOT, GRIA3 and IL1RAPL1; Carneiro et al., 2010) (see Suppl. Table 2).

Total genomic DNA was extracted from liver, muscle or testis tissues using the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to manufactures' instructions. The 12

loci were PCR amplified using the primers indicated in Suppl. Table 2. Sequencing was performed in both directions with an ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Foster City), following the ABI PRISM BigDye Terminator Cycle sequencing protocol.

2.2. Data Analyses

The nucleotide sequences were edited using BioEdit (Hall, 1999) and aligned with ClustalW (Thompson et al., 1994). Haplotypes were reconstructed for each individual using PHASE v2.1 (Stephens and Donnelly, 2003), implemented in DnaSP v5.10.01 (Librado and Rozas, 2009). The best-fit of several substitution models to each locus was assessed using jModeltest (Posada, 2008) and the Akaike information criterion (AIC). Given that the phylogenetic method to be used (see below) assumes no intra-locus recombination, a second dataset was produced using IMgc (Woerner et al., 2007), retaining the largest non-recombining blocks per locus. A balance between the number of sequences and length was looked for in order to keep at least one sequence per species per locus in the recombination-free data set.

Sequences from *Lepus granatensis* were included in the dataset as outgroup. For the X-linked loci, sequences were retrieved from Carneiro et al. (2010) and one additional specimen was newly sequenced for all loci. For the remaining loci, sequences from specimens Lgr2 and Lgr7 from Melo-Ferreira et al. (2012) were used.

Phylogenetic reconstruction was performed using the multilocus species-tree coalescent-based method implemented in *BEAST v1.8.0 (Drummond et al., 2012) both for the complete and recombination-free alignments. The Yule process and an uncorrelated lognormal relaxed clock model were used. The mutation model was set based on the AIC results of jModeltest, or if the

specific model was not implemented in *BEAST, the next most parameterized model was selected. Three independent runs of 100 000 000 generations with low autocorrelation of the Markov chain Monte Carlo (MCMC) chain, as examined using Tracer v1.5 (Rambaut and Drummond, 2007), were concatenated using LogCombiner, discarding the first 10% as burn-in. Trees were then summarized with TreeAnnotator, also part of the BEAST package. FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) was used to display the inferred species-tree.

Similarly to the strategy used by Lanier and Olson (2009), calibration of the species-tree was performed considering three possible dates of divergence between Ochotonidae and Leporidae to scale the root mean height: 31 Mya (Matthee et al., 2004), 37 Mya (McKenna and Bell, 1997; Asher et al., 2005) and 65 Mya (Bininda-Emonds et al., 2007). These alternative calibration dates and the 95% Highest Posterior Density intervals inferred for node heights, on which no prior constraints were applied, allowed considering a reasonably large range of divergence times, which reflects the uncertainties of molecular dating.

3. Results and discussion

3.1. Little influence of recombination on phylogenetic inference

In this work 11 pika species were analyzed for a total sequence length of 7506 bp (Tables 1 and Suppl. Table 2; GenBank Acc. Nrs. KP292978-KP293227; phased alignments were deposited in Dryad with doi:10.5061/dryad.bn547). Even though this is not the most comprehensive taxonomic sampling of genus performed so far, it represents the first multilocus phylogeny to date, extending the phylogenetic inference performed in other works from one (mtDNA) (Yu et al., 2000; Niu et al., 2004; Lanier and Olson, 2009; Ge et al., 2013; Lissovsky, 2014) to 12 different loci.

A substantial portion of this dataset, three loci (IL1RAPL1, UCP2 and UCP4, for which all sequences of at least one of the species were removed) and 45% of the sequence length of the remaining nine nuclear loci, was lost when retaining only the largest non-recombining blocks, resulting in a total alignment length of 2627 bp (selected mutation models for full and recombination-free datasets are shown in Suppl. Table 3). Removing recombination from the dataset resulted in the same species-tree topology when considering the highly supported nodes (>95% posterior probability), which indicates that recombination had little influence in the phylogenetic reconstruction, in agreement with the conclusions of Lanier and Knowles (2012). We thus opted to present the species-tree based on the full dataset (Fig. 1; but see alternative trees in Suppl. Figs. 1 and 2). The statistically well supported nodes of the species-tree were generally also supported by the individual gene trees sampled in the *BEAST run (Suppl. Table 4).

3.2. Major evolutionary groups support subgenera classification

The phylogenetic tree obtained in this work confirms some of the previous inferences based on the analysis of mtDNA alone. The clades represented in Fig. 1 agree with the species arrangement of the *Pika*, *Conothoa* and *Ochotona* subgenera suggested by Lanier and Olson (2009) and Lissovsky (2014). As in these works, the relationships among the three subgenera could not be reliably inferred, and a further increase of the genomic sampling may be needed to solve the polytomy observed at the base of the tree. A major advance was however made in this work associated with the relative placement of *O. pusilla* in the phylogeny, i.e. of subgenus *Lagotona* as suggested by Lissovsky (2014). While in previous works this species was either placed at the base of the genus phylogeny (Lanier and Olson, 2009; Lissovsky, 2014), or rather oddly closely related to *O. dauurica* (subgenus *Ochotona*) (Ge et al., 2013), our phylogenetic reconstruction strongly suggests

that *Lagotona* is sister to *Pika*. Discordances between gene trees and the species-tree are not surprising and may arise from many different factors, such as incomplete lineage sorting or introgression (which has been shown to particularly affect mtDNA) (Toews and Brelsford, 2012). This and/or the lack of resolution when estimating phylogenies using a relatively short mtDNA fragment may explain the previous difficulties in assessing the evolutionary history of *O. pusilla*, and highlights the increased power of our multilocus analysis.

3.3. Molecular dating of diversification and evolutionary relationships among species

Concordant inferences of node ages between the full and recombination-free datasets (Table 2 and Suppl. Table 5) were obtained. Interestingly, our estimates point to more recent divergence times than those inferred by Lanier and Olson (2009) and Ge et al. (2013), and thus fit better with paleontological estimates (see e.g. Erbajeva and Zheng, 2005), in particular using the most recent calibration point (Ochotonidae-Leporidae divergence of 31 My). McCormack et al. (2011) showed that molecular estimates of divergence dates based on a single locus tend to be overestimated by not taking into account that genetic divergence may predate speciation. This effect can be stronger for mtDNA given its lower effective population size and consequent tendency to more rapidly sort lineages. Our multilocus approach thus seems to provide a more robust estimate of dates of diversification in the genus *Ochotona*.

According to fossil records, the formation of the steppe zone in Eurasia which resulted from a global environmental change by the end of late Miocene allowed an explosive radiation of *Ochotona* (see Erbajeva and Zheng, 2005). Our estimates coincide with this assessment, placing this radiation 6.5-14 Mya (depending on the calibration date used) with the lower bound of the 95% Higher Posterior Density (HPD) at ~4 Mya (node B; Fig. 1; Table 2). In addition, our inference

suggests that the divergence of *Lagotona* from subgenus *Pika* must have occurred shortly after, 4-9 Mya (node D; Fig. 1; Table 2).

Within subgenus *Pika*, the North American *O. princeps* was the first to diverge from Asian pikas at Miocene/beginning of Pliocene around 3-7 Mya (node E; Fig. 1; Table 2). Of the remaining six species of *Pika* analysed here five are morphologically similar (*O. hyperborea*, *O. mantchurica*, *O. hoffmanni*, *O. alpina* and *O. turuchanensis*) and form the alpina group. Our phylogeny however did not support the monophyly of this group, because it was not able to resolve the polytomy that includes the alpina group and *O. pallasi* (node F; Fig. 1). Whether this is due to poor phylogenetic resolution from our 12 loci must be assessed in the future with an increased genetic sampling and larger sample sizes of these species. It nevertheless shows that the monophyly of the alpina group suggested by previous mtDNA-based phylogenies (e.g. Lanier and Olson, 2009; Lissovsky, 2014) should be interpreted with caution. The same occurs at a finer scale. For example, the polytomy at the base of the diversification of *O. hyperborea*, *O. mantchurica* and *O. hoffmanni* (node G; Fig. 1) could not be solved. These are very closely related species that we estimated to have separated 0.8-2 Mya, with lower bound of the 95% HPD at 400 kya (Table 2). Likewise, *O. alpina* and *O. turuchanensis* were confirmed to be very closely related and to have putatively separated very recently, 278-583 kya (node H, Fig. 1; Table 2), with lower 95% HPD bound at 127 kya. We note that we sampled only a few variants per species and thus cannot confirm the levels of genetic isolation of these taxa. Only a devoted work with a thorough sampling of each of the entities would allow properly capturing the variance of the evolutionary process of divergence (for example in an isolation-with-migration framework; Hey, 2010), shed light onto the degree of genetic isolation and contribute to the discussion of the validity of the specific status of these taxa.

3.4. Conclusion and future prospects

This work provides a robust view of the evolutionary history of the major groups of the genus *Ochotona*. Previous molecular phylogenies of the genus were limited to the analysis of a single locus – mtDNA – which could have resulted in misleading inferences. Our results, based on 12 independent loci, provide new insights on the phylogenetic position of subgenus *Lagotona*, sister to *Pika*, and advise caution when considering the monophyly of the alpina group. This work also made progress in the understanding of the time-frames of the major diversification events within the genus, which tended to be overestimated in mtDNA-based inferences.

The complete clarification of the phylogeny and systematics of genus *Ochotona* remains, however, a challenge that should be addressed at both broader and finer evolutionary scales. At a broader scale, increasing the number of sampled species (here we sampled about a third) is fundamental to clarify the composition of the major evolutionary groups and identify the sequence and timing of speciation events in the genus. The methods and molecular markers used in this work appear suitable to address this issue. At a finer scale, larger intraspecific sampling of complexes of closely related species (e.g. the alpina group or the American species) is needed to properly infer the levels of genetic isolation between the forms and contribute with genetic information to the criteria for species classifications. The molecular markers used in this work are also important contributions for such studies. It must however be considered that the recognition of species status should not rely on a simplistic genetic formula and must take into account other sources of information on the biology and history of the organisms in question.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Table 1: Species, geographic location and ID of the specimens newly sequenced in this study

Species	Common name	Continent	Code	Sample Locality	Specimens id.[1]
Ochotona alpina	Alpine pika	Asia	Oal1	Republic of Gorno-Altay, Russia	S-183539
			Oal2	Altayskiy Kray, Russia	S-171510
Ochotona dauurica	Daurian pika	Asia	Oda1	Zabaikalskiy Kray, Russia	S-182045
			Oda2	Zabaikalskiy Kray, Russia	S-175916
			Oda3	Arahangay Aymak, Mongolia	S-183342
Ochotona hoffmanni	Hoffmann's pika	Asia	Oho1	Zabaikalskiy Kray, Russia	S-180456
			Oho2	Zabaikalskiy Kray, Russia	S-180455
Ochotona hyperborea	Northern pika	Asia	Ohy1	Yakutia Republic, Russia	S-183415
			Ohy2	Krasnoyarskiy Kray, Russia	S-167268
Ochotona mantchurica	Manchurian pika	Asia	Oma1	Zabaikalskiy Kray, Russia	S-182046
			Oma2	Zabaikalskiy Kray, Russia	S-178619
Ochotona pallasi	Pallas's pika	Asia	Opa1	Bayan-Ulegey Aymak, Mongolia	S-183345
			Opa2	Republic of Gorno-Altay, Russia	S-183540
Ochotona princeps	American pika	North America	Opr1	Unknown	-
Ochotona pusilla	Steppe pika	Asia	Opu1	Orenburgskaya Oblast'. Russia	S-181302
			Opu2	Orenburgskaya Oblast', Russia	S-181301
			Opu3	Orenburgskaya Oblast', Russia	S-181303
Ochotona rufescens	Afghan pika	Asia	Orf1	Khorasan, Iran	S-178637
			Orf2	Khorasan, Iran	S-178636
Ochotona rutila	Turkestan red pika	Asia	Ort1	Kashkadarya Region, Uzbekistan	S-181326
			Ort2	Kashkadarya Region, Uzbekistan	S-181325
Ochotona turuchanensis	Turuchan pika	Asia	Otu1	Irkutskaya Oblast', Russia	S-171587
			Otu2	Krasnoyarskiy Kray, Russia	S-162967

[1]Code from the Zoological Museum, Moscow State University, Moscow, Russia.

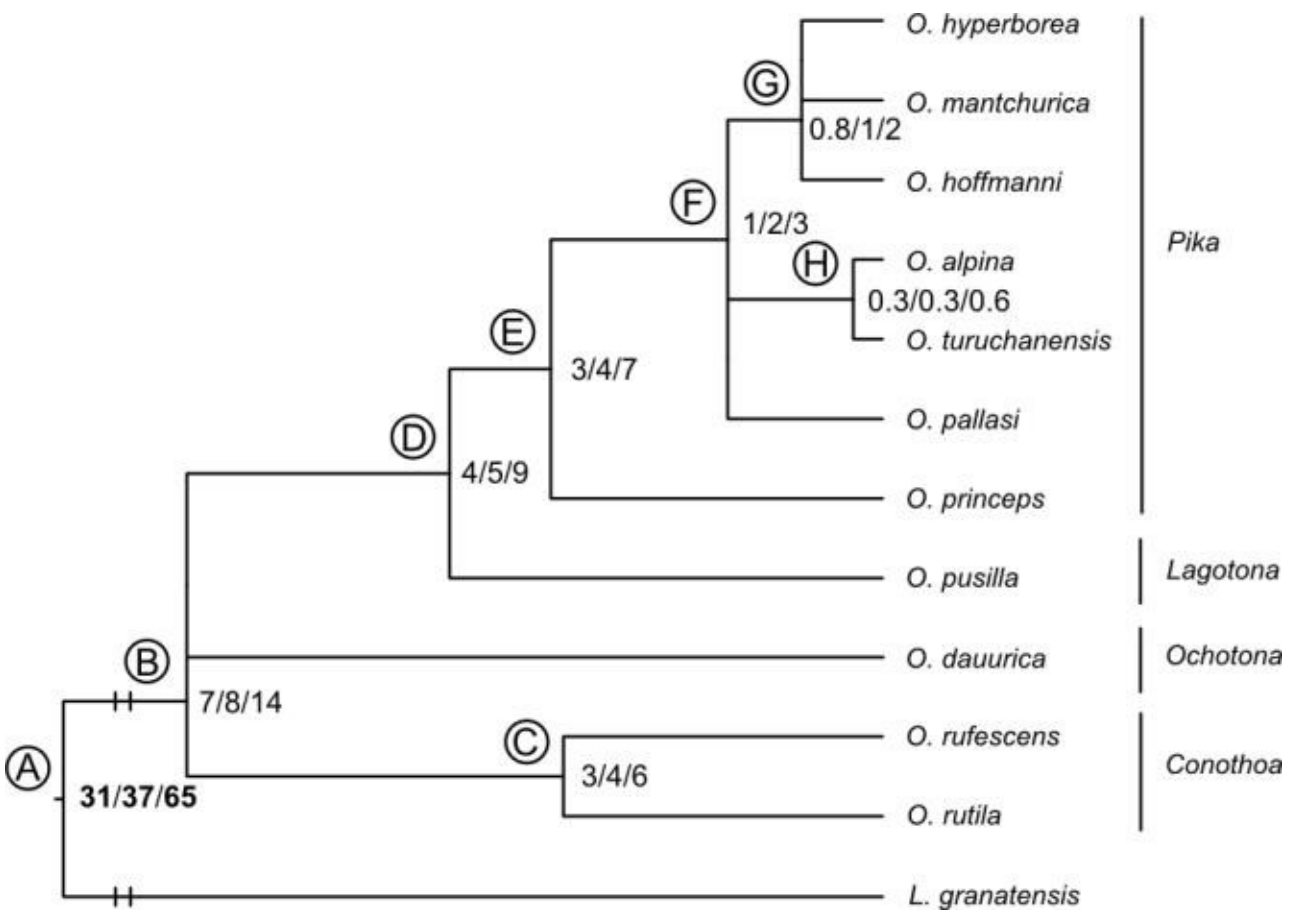
Table 2: Inferred node ages (in million years) based on three dates of Ochotonidae-Leporidae divergence and the full dataset (see Fig. 1 for node correspondence; 95% Highest Posterior Density intervals in parenthesis).

Calibration (Node A)	Node B	Node C	Node D	Node E	Node F	Node G	Node H
31 Mya	6.569 (4.158, 9.157)	3.015 (1.828, 4.351)	4.090 (2.546, 5.723)	3.133 (1.916, 4.469)	1.462 (0.857, 2.104)	0.765 (0.442, 1.132)	0.278 (0.127, 0.443)
37 Mya	7.841 (4.962, 10.929)	3.599 (2.181, 5.193)	4.881 (3.038, 6.830)	3.740 (2.287, 5.334)	1.745 (1.023, 2.511)	0.913 (0.527, 1.351)	0.332 (0.152, 0.529)
65 Mya	13.774 (8.717, 19.200)	6.322 (3.832, 9.122)	8.575 (5.338, 11.999)	6.570 (4.018, 9.370)	3.066 (1.797, 4.412)	1.604 (0.926, 2.374)	0.583 (0.266, 0.930)

Figure Legend

Fig. 1: Species-tree of *Ochotona* inferred with *BEAST from the full dataset indicating subgenera according to Lissovsky (2014) and this work. Numbers next to nodes indicate posterior probabilities. Nodes with posterior probability below 95% were collapsed (see full phylogeny in Suppl. Fig. 1 and recombination-free phylogeny in Suppl. Fig. 2). Inferred mean node ages calibrated with three estimates of Leporidae-Ochotonidae divergence (node A) are shown in Tables 2 and Suppl. Table 5.

Figure 1:


O. hyperborea
O. mantchurica
O. hoffmanni
O. alpina
O. turuchanensis
O. pallasi
O. princeps
O. pusilla
O. dauurica
O. rufescens
O. rutila
L. granatensis
Pika
Lagotona
Ochotona
Conothoa
G
0.8/1/2
F
1/2/3
H
0.3/0.3/0.6
E
3/4/7
D
4/5/9
B
7/8/14
C
3/4/6
A
31/37/65