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Inferring divergence times within pikas (*Ochotona* spp.) using mtDNA and relaxed molecular dating techniques

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ABSTRACT

Although several studies have recently addressed phylogenetic relationships among Asian pikas (*Ochotona* spp.), the North American species have been relatively neglected and their monophyly generally unquestioned or assumed. Given the high degree of intraspecific diversity in pelage and call structure, the recent identification of previously unrecognized species of pika in Asia, and the increasing evidence for multiple trans-Beringian dispersals in several small mammal lineages, the monophyly of North American pikas warrants reexamination. In addition, previous studies have applied an externally calibrated rate to examine the timing of diversification within the genus. This method has been increasingly shown to return results that, at the very least, are overly narrow in their confidence intervals, and at the worst can be entirely spurious. For this study we combined GenBank sequences from the mitochondrial genes cyt *b* and ND4 with newly generated sequence data from *O. hyperborea* and *O. collaris* to investigate the origin of the North American lineages and the timing of phylogenetic diversification within the genus *Ochotona*. Specifically, we address three goals (1) summarize and reanalyze the molecular evidence for relationships within the genus using statistically supported models of evolution; (2) add additional sequences from *O. collaris* and *O. hyperborea* to rigorously test the monophyly of North American pikas; (3) examine the timing of the diversification within the genus using relaxed molecular clock methods. We found no evidence of multiple trans-Beringian dispersals into North America, thereby supporting the traditional hypothesis of a single invasion of North America. We also provide evidence that the major splits within the genus occurred in the Miocene, and the Nearctic pikas diverged sometime before the Pleistocene.

1. Introduction

Pikas (Lagomorpha: Ochotonidae; *Ochotona*) comprise 30 species of territorial, small-bodied herbivores currently found in the northern hemisphere (Smith et al., 1990; Hoffmann and Smith, 2005). Thought to have diverged from their sister lineage Leporidae (rabbits and hares) sometime after the K/T boundary (65 mya; but see Bininda-Emonds et al., 2007), pikas are one of the least speciose of any of the families in the superorder Glires (Orders Lagomorpha and Rodentia). Pikas are generalist herbivores adapted to steppe and alpine environments. Extant species (genus *Ochotona*) exhibit high intraspecific variation in pelage and vocalizations but few differences between species (Corbet, 1978; Smith et al., 1990; Erbajeva, 1994). Consequently, their taxonomy has been, and continues to be, poorly resolved (Hoffmann and Smith, 2005).

Two recent molecular studies have explored phylogenetic relationships within *Ochotona* using DNA sequence data. The first (Yu et al., 2000) sampled two mitochondrial genes (1383 bp of ND4 and 1140 bp of cytochrome *b*) from 19 extant species, focusing mainly on the timing and divergence of Asian *Ochotona*. Yu et al. (2000) recovered three main subgroups of pika: a northern subgroup, a shrub–steppe dwelling subgroup, and a mountain subgroup. Largely based on this work, *Ochotona* was grouped into three subgenera (Table 1) by Hoffmann and Smith (2005). While the Yu et al. (2000) analysis provides an important foundation for understanding pika systematics, it included very few intraspecific samples from broadly separated localities and failed to include a number of potentially important species, particularly *O. collaris* (North America) and *O. pusilla* (Eurasia). A second molecular study was recently conducted by Niu et al. (2004), incorporating more taxa but shorter fragments of cyt *b* (~402 bp). As ochotonid taxonomy can be complex and species often misidentified (Smith et al., 1990), the inclusion of additional species and intraspecific samples is important. However, larger taxonomic sampling frequently requires larger molecular datasets to provide sufficient phylogenetic

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Table 1
Subgenera and fossil record of extant Ochotona species.

Subgenus[a]	Species	First known fossil occurrence
Conothoa	*O. brookei*	
	O. erythrotis	Late Pliocene[b]
	O. forresti	
	O. gaoligongensis	
	O. gloveri	
	O. himalayana	
	O. iliensis	
	O. koslowi	Pleistocene[c]
	O. ladacensis	
	O. macrotis	Middle Pleistocene[d]
	O. muliensis	
	O. nigritia	
	O. roylei	
	O. rutilla	
Ochotona	*O. cansus*	
	O. curzoniae	
	O. dauurica	Middle Pleistocene[e]
	O. huangensis	
	O. nubrica	
	O. pusilla	Late Pliocene[e]
	O. rufescens	Middle Pleistocene[e]
	O. thibetana	Late Pleistocene[c]
	O. thomasi	
Pika	*O. alpina*	Middle Pleistocene[e]
	O. argentata	
	O. collaris	Late Pleistocene[f]
	O. hoffmanni	
	O. hyperborea	Late Pleistocene[e]
	O. pallasi	
	O. princeps	Middle Pleistocene[g]
	O. turuchanensis	

[a] Hoffmann and Smith (2005).
[b] Cai (1989).
[c] Li et al. (2006).
[d] Erbajeva and Zheng (2005).
[e] Erbajeva (1994).
[f] Harington (1978).
[g] Mead (1987).

resolution. Recent morphological (Lissovsky, 2003) and molecular (Lissovsky et al., 2007) studies have also been conducted on the Palearctic members of the subgenus *Pika*. Phylogenetic analyses by Lissovsky et al. (2007) incorporated greater sampling within the *O. alpina–hyperborea* complex, resulting in the recognition of the morphologically, ecologically, and genetically distinct species, *O. turuchanensis*. Lissovsky (2003) and Lissovsky et al. (2007) also suggested the presence of at least one additional species, *O. scorodumovi*, which may be conspecific with *O. mantchurica*.

1.1. Nearctic pikas

In North America, two allopatric species of pika are currently recognized—*O. collaris* and *O. princeps*—which have been traditionally defined on the basis of geographic isolation, size of auditory bullae, and pelage characteristics (Nelson, 1893; Hall, 1951; Broadbooks, 1965; MacDonald and Jones, 1987). The many similarities between the two species led some authors to synonymize *O. collaris* and *O. princeps*, along with the Palearctic *O. hyperborea*, with the Palearctic species *O. alpina* (e.g., Broadbooks, 1965; Corbet, 1978). Weston (1981) used morphometric data to show distinct differences between the North American taxa, with *O. princeps* more closely resembling the Asian species. In terms of intraspecific variation, *O. princeps* includes 36 recognized subspecies and a much patchier distribution then the monotypic *O. collaris* (Hall, 1981). This may be an artifact of disproportionate research efforts over the past century, as *O. princeps* has been more frequently studied than *O. collaris* (MacDonald and Jones, 1987; Smith and Weston, 1990). While subspecific differentiation within *O. collaris* has been suggested (Baker, 1951), it has yet to be thoroughly investigated. It may also reflect true levels of intraspecific variation, possibly resulting from events of historical population fragmentation within *O. princeps* or a more recent bottleneck within *O. collaris* (Hafner, 1994; Hafner and Sullivan, 1995).

1.2. Biogeography and fossil history

Currently, pika diversity is highest in Asia (28 species), with only two species in North America. The current distribution and diversity of the pikas is a fraction of that seen during their peak in the Miocene, with fossils representing multiple genera known from localities as far apart as north Africa, eastern North America, and western Europe (Dawson, 1967). Pleistocene records exist for steppe pika (*O. pusilla*) in Great Britain, although this species is now known only from the central Russian steppes and northern Kazakhstan (Erbajeva, 1994; Fisher and Yalden, 2004; Smith et al., 1990).

Pikas are known from North American localities as early as the Miocene (in Oregon; Shotwell, 1956), and as far south as California, as far east as Virginia in the Pleistocene (Kurtén and Anderson, 1980; Erbajeva, 1994; Mead and Grady, 1996). Several North American species have been described, including a relatively large form (*O. whartoni*) from Alaska and the Yukon Territory (Mead, 1987; Guthrie and Matthews, 1971). A smaller species, possibly *O. collaris*, is thought to have been sympatric (although perhaps not contemporary) with *O. whartoni* (now extinct; Guthrie and Matthews, 1971; Harington, 1978; Weston, 1981; Mead, 1987). Distribution maps of *O. princeps* and *O. collaris* have been used to suggest allopatric speciation in separate refugia during the Wisconsinan glaciation (Guthrie, 1973; Harington, 1978). This scenario would imply that *O. collaris* and *O. princeps* diverged within North America after a single dispersal across the Bering Land Bridge from Asia. However, similar assumptions about the Alaska marmot (*Marmota broweri*) and the hoary marmot (*M. caligata*), also North American alpine specialists with similarly allopatric distributions, proved incorrect, as molecular data suggest that the Alaskan marmot is the result of an independent colonization and is actually more closely related to Asian species (Steppan et al., 1999).

1.3. Current objectives

Pikas exhibit a great deal of intraspecific morphological diversity and relatively low interspecific diversity, making it unreasonable to use a single sample per putative species as a proxy for that species. An increasing number of studies have demonstrated the importance of sampling multiple individuals from multiple species to understand the evolutionary history and taxonomic limits of a species (Peters et al., 2005). Using a single individual, or geographically clumped sample from multiple individuals, could fail to capture paraphyly or polyphyly resulting from introgression, hybridization, and/or incomplete lineage sorting of ancestral polymorphisms (Peters et al., 2007). As many talus-dwelling species live in patchily distributed habitat with disjunct populations (Smith et al., 1990), they may be likely to contain cryptic lineages. Several of the most recently described species of pika are talus-dwelling, and very geographically restricted (e.g., *O. iliensis*, Li and Smith, 2005; *O. argentata*, Erbajeva and Ma, 2006). Little is known about the potential for pika species to hybridize, but rabbits and hares show evidence of introgression or hybridization between species (Alves et al., 2003). While denser taxonomic sampling is important, it necessitates increasing the number of characters to improve resolution and nodal support (Jansa et al., 2006).

Statistical and computational phylogenetic methods have greatly improved over the past few years, and we sought to utilize

those advances to better test unresolved issues in pika phylogeny. Neither the Yu et al. (2000) nor the Niu et al. (2004) study employed a statistically supported model of evolution. Simpler models are known to perform poorly on trees with short internodes and relatively long external branches (Felsenstein, 1978). In addition, they are likely to underestimate the amount of evolution that has occurred on a tree and subsequently bias the branch lengths downward (Yang, 2006). Explicit models utilize parameters that can be captured and compared through hierarchical likelihood ratio tests (hLRT), the Akaike Information Criterion (AIC), or the Bayesian Information Criterion (BIC) in order to choose the model that best fits the data (Posada and Crandall, 1998; Posada and Buckley, 2004). Accounting for rate variation between sites is critical to correctly estimating branch lengths, and correctly estimating branch lengths is essential for accurately inferring time since divergence.

Many systematic studies construct phylogenies and apply molecular clocks to their data without checking for clock-like behavior in their datasets (Peterson, 2006). Despite the general consensus that the dates obtained from misusing these techniques are probably somewhat incorrect, authors continue to apply molecular clocks in this fashion because it provides a genealogical comparison to geological events. Applying an externally calibrated rate (e.g., 2% divergence per million years for mtDNA) fails to account for rate variation and, when strictly applied, can vastly underestimate the uncertainty around a particular divergence date (Thorne, pers. comm., HCL 2007; Pulquério and Nichols, 2007). While using the fossil record to calibrate nodes within a phylogeny is generally agreed to be the most appropriate method, users need to exercise caution to correctly apply dates and incorporate error estimates (Graur and Martin, 2004; Benton and Donoghue, 2007; Ho, 2007). Relaxed molecular clock techniques provide increasingly sophisticated models for estimating a range of plausible divergence dates (Sanderson, 2002; Drummond et al., 2006).

Two of the previous studies of pika systematics using molecular data have assumed a strict molecular clock (Yu et al., 2000; Niu et al., 2004). Each of these studies calculated point estimates of divergence dates between some taxa, most of which fall within the Pleistocene. Several studies (Hoberg, 2005; Waltari et al., 2007) have used dates based on these studies, so we are particularly interested in determining their validity. Rather than applying a strict molecular clock or calibrating with a single point estimate, we are interested in determining the range of plausible divergence dates supported by the data to test the hypothesized Pleistocene divergence of North American pikas (proposed by Guthrie, 1973). This hypothesized Wisconsinan split in the Nearctic pikas has been cited by many authors (e.g., Harington, 1978; Weston, 1981; MacDonald and Jones, 1987) and is consistent with the divergence dates estimated by Niu et al. (2004), who applied a strict molecular clock. An increasing number of studies are finding that Pleistocene climate changes caused within-species phylogeographic structure but that most speciation events predate the period (Near et al., 2003).

2. Methods

2.1. Taxon and marker selection

We combined sequences from eight published studies with additional sequences generated specifically for this study to better understand the origin of the Nearctic pikas and the phylogenetic relationships within *Ochotona*. Our aim was to integrate the additional taxonomic sampling of Niu et al. (2004) and other GenBank contributors, the high degree of intraspecific sampling of Lissovsky et al. (2007), and the longer sequences used by Yu et al. (2000). We augmented the existing GenBank data (101 individuals of *Ochotona*, 2 leporid outgroups; Appendix A) with additional sequences from pikas on either side of the Bering Strait (Appendix B). We placed particular emphasis on including samples of *O. collaris* from throughout its range, and samples of *O. hyperborea* from localities closest to the Bering Strait.

We analyzed two complementary datasets, one with greater taxon sampling (cyt *b*) and one with fewer taxa but more characters (cyt *b* + ND4). For the cyt *b* dataset, we included most available GenBank *Ochotona* sequences, including several that have recently been called into question. Lissovsky et al. (2007) highlighted potential errors in some published cyt *b* data and, for that reason as well as concerns over specimen misidentification, excluded all sequences generated by Niu et al. (2004). We chose to include the sequences from Niu et al. (2004) because we feel that their analysis in a phylogenetic framework provides important information. Lissovsky et al. (2007) also excluded an unspecified 153 bp region of another sequence because it contained several unique amino acids (*O. alpina*; GenBank Accession AF273009; Yu et al., 2000). While we agree that there are suspicious differences in this particular sequence, a lack of detailed amplification and sequencing strategy (in Yu et al., 2000) meant that we were unable to rigorously test for potential chimerism. However, BLAST results for the general region of AF273009 questioned by Lissovsky et al. (2007) suggested an *Ochotona* origin (results not shown). Additional corroboration comes from an identical haplotype (to AF273009) recovered by Niu et al. (2004) from a different specimen of the same species. Conversely, an *O. pallasi* sequence (DQ335521) published on GenBank by Lissovsky et al. (2007), but inexplicably excluded from their published paper, was excluded from this analysis because a BLAST search returned no similar *Ochotona* sequences.

2.2. DNA amplification and sequencing

DNA was extracted from frozen tissues using the PureGene kit (Gentra Systems Inc.) following the manufacturer's Animal Tissue Protocol. Purified DNA was resuspended in 100 μL DNA Hydration Solution, and a 1:10 working dilution was used in PCR reactions. Amplification followed standard PCR protocols at a magnesium concentration of 1.5 mM, with 30 cycles of denaturation, annealing (50 °C), and extension (using primers marked with a superscript in Table 2). Primers were designed with reference to the complete mitochondrial genomes of *O. collaris* (AF348080) and *O. princeps* (AJ537415). PCR products were purified with Exo/SAP (USB Corp.) and sequenced directly with amplification (external) primers and internal primers and 0.5 μL of ABI BigDye v3.1 terminators (Applied Biosystems) on an ABI 3100 automated sequencer. Double-stranded sequences were generated for all samples, with at least 50 bp of overlap between adjacent fragments. Sequences were assembled and edited using Sequencher 4.5 (GeneCodes Corp.). All sequences generated in this analysis, along with their specimen

Table 2
Primers.

Name	Sequence (5′–3′)
CB-HLF1[a]	CCACCGTTGTAGTTCAACT
CB-HLiF2	AGCCACCCTAACTCGATTCT
CB-HLiR2	AGCCTGTTTCGTGGAGGAAGAGTA
CB-HLR1[a]	GGTTTACAAGACCAGGGTA
ND4-HLF1[a]	CCAACACATACGGCATAGACTA
ND4-HLiF2	ATCACCCGATGAGGTAACCAAACA
ND4-HLiF3	TGGCACTCGTAATTGTCGCAA
ND4-HLiR3	TTGTGGATGTAGAGTAGGGCTA
ND4-HLiR2	TTCGGCTGTGGATACGTTCATA
ND4-HLR1[a]	CGGTGGATAAGAGGTTGTTA

[a] External primers.

voucher numbers, have been deposited in GenBank (Accession Nos. EU549736–EU549756; Appendix B).

2.3. Phylogenetic analysis

GenBank sequences varied in length from 317 to 1140 bp and from 820 to 1383 bp (cyt *b* and ND4, respectively; Appendix A). Sequences generated specifically for this study were complete (1140 bp for cyt *b* and 1383 bp of ND4 after primer sequences were excised). We aligned sequences by eye in MacClade (Maddison and Maddison, 2000). Phylogenetic analyses were first conducted on each gene separately for data exploration purposes. However, only results for cyt *b* and combined gene (cyt *b* + ND4) analyses are presented and discussed.

We used GARLI (v. 0.95, Zwickl, 2006) to conduct heuristic tree searches and bootstrapping under the maximum likelihood criterion. GARLI employs a genetic algorithm to find the topology and parameters compatible with the data that yield the highest likelihood. Nucleotide substitution models were chosen using the Akaike Information Criterion in ModelTest 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). Analyses were conducted using the model of nucleotide substitution suggested by ModelTest, allowing the parameters within that model to be estimated by GARLI, and using an initial starting topology created using the neighbor-joining method in PAUP* (Swofford, 2002). Likelihood searches were run using the default parameters for run termination and optimization. Each bootstrap pseudoreplicate was run until $-\ln L$ values converged (changing less than 0.02) for 5000 generations. PAUP* was then used to calculate nodal support by computing a consensus tree of the 100 bootstrap pseudoreplicates.

Bayesian posterior probabilities and topologies were estimated using MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For each single-locus dataset (cyt *b* and ND4), we performed separate Bayesian analyses (BA), one in which parameters were applied to the whole gene with a proportion of invariant sites and a gamma rate distribution. Combined gene datasets were analyzed using a GTR + I + Γ model, partitioned between genes, and a GTR model partitioned by codons. A Bayes factor comparison showed that for the combined datasets the codon-partitioned model was decisively better (K = 457; 1000 $-\ln L$ units) than the gene-partitioned model, and only the results of the codon-partitioned model will be discussed. Tree searches were conducted using four chains, three of which were heated, allowed to run for 10 million generations and sampled every 1000 generations. The first 200 trees (representing 200,000 generations) were discarded as burn-in, using the plots of $-\ln L$ scores against generation as a guide.

2.4. Molecular clock calibration

While there are a fair number of fossil ochotonids (including but not limited to fossils of extant taxa listed in Table 1), the phylogenetic placement of most fossil taxa within the genus *Ochotona* is uncertain. There are no clear morphological synapomorphies uniting subgenera within *Ochotona* (Yu et al., 2000; Hoffmann and Smith, 2005). However, there are some fairly accurate estimates that have been proposed for the divergence between Leporidae and Ochotonidae. Dates for this node vary depending on whether they are based on fossil evidence alone or molecular trees calibrated with multiple fossil taxa (e.g., McKenna and Bell, 1997 vs. Bininda-Emonds et al., 2007). Fossil evidence alone suggests that the divergence between Leporidae and Ochotonidae occurred at or before 37 mya (based upon the first fossil occurrence of Ochotonidae in the late Eocene; McKenna and Bell, 1997). The 37 mya divergence also coincides with recent fossil- and molecular-based divergence estimates within Glires (Asher et al., 2005). We chose to contrast the divergence dates within *Ochotona* using a relaxed molecular clock method and a 37 mya leporid–ochotonid split with those estimated under alternative models of rabbit–pika divergence. This method produces a reasonable range of plausible divergence dates for each node within *Ochotona* and excludes those dates that are not compatible with any of the likely rabbit–pika divergences. One of the most recent dates proposed for the leporid–ochotonid divergence is around 31 mya (inferred using multiple fossil calibrations, nuclear DNA, and a relaxed molecular clock approach; Matthee et al., 2004). While this date appears to contradict fossil evidence, we feel that it provides a lower bound of estimates for hypothesis testing to account for potential taxonomic and stratigraphic errors in calibration. On the other extreme, a recent study employing a supertree approach and multiple fossil calibration points suggests the leporid–ochotonid split may have occurred at or before the K/T boundary (60–70 mya; Bininda-Emonds et al., 2007). This 65 mya model provides an upper bound, encompassing dates resulting from an Early Eocene rabbit–pika split as has been suggested by recently described fossil Lagomorpha (Rose et al., 2008) and other molecular studies (Springer et al., 2003). Instead of choosing one of these dates, or combining all three into one uniform (and fairly noninformative) prior, we conducted three separate analyses using a different calibration point for the root node (31, 37, and 65 mya) to determine the range of plausible divergence dates supported by the data for the major splits within *Ochotona*.

2.5. Molecular dating methods

We used a relaxed molecular phylogenetics technique, implemented in the program BEAST (Drummond et al., 2006), to simultaneously estimate phylogeny and divergence times within *Ochotona*. For the purposes of estimating divergence dates, we analyzed a pruned version of the combined gene dataset that minimized the number of intraspecific samples (see Appendices A and B). Population-level samples are undergoing mutation/fixation at different rates from species-level samples and tend to have very short branches that complicate rate estimation (Ho et al., 2005). BEAST runs were conducted using a GTR model of evolution, with the dataset partitioned by codons and rates unlinked between partitions. We used a relaxed lognormal molecular clock model, which has been shown to generate accurate estimates of rates with narrow Highest Posterior Density intervals (Drummond et al., 2006; Ho, 2007). We used a Yule prior on the tree to simulate the process of speciation. Operators were tuned with successive runs of the program under the chosen model, with slight changes to the scaling factors until the Effective Sample Size for each parameter exceeded 200 (as recommended by Drummond and Rambaut, 2007). Once optimum operator scaling was achieved, we re-ran the analysis six times (twice for each potential calibration point corresponding to the lagomorph–ochotonid split) for 10 million generations. A 10% burn-in was discarded from the beginning of each run, and all samples were examined in Tracer (Rambaut and Drummond, 2004) to verify an effective sample size exceeding 200 for all parameters being estimated. Independent runs for each calibration point were then combined to yield parameter estimates for divergence dates.

3. Results

3.1. Model selection and phylogenetic results

ModelTest found that the preferred model of evolution for the cyt *b* dataset, according to both the hierarchical likelihood ratio test and the AIC, is the General Time Reversible model (GTR) with a gamma-distributed shape parameter (α = 1.0656) and a proportion of invariant sites (I = 0.5199). The GTR + Γ + I was also selected for the ND4 matrix (α = 1.1584; I = 0.4772) and the combined matrix (α = 1.1481; I = 0.5077).

When all available cyt *b* samples for *Ochotona* were analyzed, we generally recovered monophyly of the three main subgenera found by Yu et al. (2000) (Fig. 1), with high support (74 ML BS, 0.94 posterior probability) for subgenus *Pika* and moderate to low support for the other subgenera. For the combined dataset, topologies from all analyses were the same, or non-conflicting. The placement of the root was variable between analyses. While the Bayesian cyt *b* + ND4 analyses rooted between the *Conothoa* and *Pika* + *Ochotona* clades, the ML topology resulted in an alternative rooting on the branch leading to *O. erythrotis* at the base of *Conothoa* (Fig. 2, black dot). The cyt *b* tree tends to root on *O. pusilla*, with *O. huangensis* is weakly supported as being basal to the *Pika* + *Conothoa* clade, but there was some alternative support for rooting within *Conothoa* (Fig. 1, black dots). Subgenera *Ochotona* and *Pika* (Yu et al.'s shrub–steppe and northern subgroups, respectively) were strongly supported (85% BS, 99% PP) as being sister to one another in all cyt *b* + ND4 analyses. When the cyt *b* dataset is analyzed, the relative placement of the subgenera is not well resolved.

Under most algorithms and models (partitioned by codon and unpartitioned), the placement of *O. pusilla* is not well resolved (Fig. 1). In some cases it is recovered as being basal, and therefore outside the three described subgenera, but this relationship never received strong (>70 posterior probability or bootstrap) support. The placement of *O. huangensis* is poorly resolved in all reconstructions. While it was (54% BS, 84 PP) recovered as sister to the shrub–steppe group (subgenus *Ochotona*) in the combined dataset, it was reconstructed as being sister to *O. pusilla* in the maximum likelihood tree but with low support (<50 BS, <75 PP). *Ochotona pallasi helanshanensis*, thought to be synonymous with *O. argentata* (Erbajeva and Ma, 2006), is nested within *O. pallasi*. The sequence identified as *O. annectens* (AF273008; Yu et al., 2000) groups most closely with several *O. curzoniae* sequences (Fig. 1) and is not supported as being closely allied to *O. dauurica* despite its putative subspecific status (*O. dauurica annectens*; Hoffmann and Smith, 2005). The questionable *O. alpina* sequences (starred in Fig. 1) were recovered on a particularly long branch as being sister to the rest of *O. alpina*.

Out of the 15 species for which more than one sample per species was included, 10 were recovered as monophyletic. The remaining five were not recovered as monophyletic in any of our analyses. Most of these were within the subgenus *Ochotona* and involved burrowing species. Within two species, *O. princeps* and *O. hyperborea*, relatively deep intraspecific divergences were inferred, comparable to or exceeding divergence between other species of *Ochotona* (as measured by branch length). Despite the additional samples of *O. hyperborea* and *O. collaris* from either side of the Bering Strait, considerable support was still recovered for the monophyly of the North American taxa. Under all algorithms and all models, *O. collaris* was strongly supported as the sister to *O. princeps*, and the previously recovered relationships within the subgenus *Pika* (*O. pallasi* sister to the *alpina–hyperborea* complex) were unequivocally supported in all analyses.

3.2. Molecular dating

All three models of leporid–ochotonid divergence supported a Miocene common ancestor for the extant *Ochotona* (Table 3). While both the 31 and 37 mya models supported Middle to Late Miocene subgeneric diversification, the 65 mya model extends this to the Early to Middle Miocene. Substitution rates were likewise scaled depending on the timing of the leporid–ochotonid split, with the 31 mya model favoring a rate ranging from 1.7% to 2% million years, the 37 mya root model suggested a rate of 1.4–1.7% million years, and a rate of 0.2–1% mya under the 65 mya model. The older the node, the greater the discrepancies between dates inferred under a strict molecular clock and those resulting from a relaxed molecular clock approach (Fig. 4).

In both the 31 and 37 mya scenarios, the major diversifications within the subgenus *Conothoa* were Late Miocene to Early Pliocene (Fig. 2). These models supported a more recent common ancestor (MRCA) for most of the members of the subgenus *Ochotona*, with the majority of the support being for a Pliocene or Pleistocene diversification. The 65 mya scenario places most of the major divergences within the genus *Ochotona* during the Miocene, with some of the 95% credibility intervals (CI) for the MRCA of the genus occurring in the late Oligocene to early Miocene. None of the three analyses recovered support (within the 95% CI set of trees) that included a common ancestor of *O. collaris* and *O. princeps* in the Pleistocene (Table 3; Fig. 3).

4. Discussion

4.1. Model selection, branch lengths, and taxonomic implications

In general, incorporating a statistically supported model of evolution resulted in changes in branch length, with topological changes resulting around long branches with short internodes (e.g., within subgenus *Conothoa*). In their maximum parsimony tree, Niu et al. (2004) recovered strong bootstrap support for an *O. alpina* + *O. pallasi* clade, which was not recovered using any other methods in their paper or by other authors (e.g., Yu et al., 2000; Lissovsky et al., 2007, this study). We believe this to be the result of long-branch attraction, and were able to replicate their results using a dataset trimmed to the same 402 bp and subjected to parsimony analysis (results not shown). This example highlights the need for the use of both appropriate models of evolution and large numbers of characters. Incorporating a more statistically supported, and hopefully correct, model of sequence evolution is also particularly important in cases where timing of events is being tested or even discussed. Trees incorporating these models were more than twice as long as those relying on more simplistic models (Yu et al., 2000; Niu et al., 2004). Although these models make more assumptions, we feel that their use is statistically justified through higher likelihood scores and empirically justified by the high probability of multiple changes to the same nucleotide in the mitochondrial genome (Posada and Crandall, 1998; Posada and Buckley, 2004).

The placement of the root is variable, affecting phylogenetic support for the inclusion of two taxa (*O. pusilla* and *O. huangensis*) in the subgenus *Ochotona*, where they were placed by Hoffmann and Smith (2005). The position of the root in our three datasets affects the interpretation of phylogenetic results, and we feel that much work remains to determine species limits and phylogenetic relationships within *Ochotona*. Pleistocene fossils, a broad distribution, and a karyotype of $2N = 68$ (identical to the North American taxa) have all been used to justify the basal position of this taxon within the pikas (Erbajeva, 1994; Niu et al., 2004). However, *O. pusilla* has only ever been weakly supported as sister to the other *Ochotona*. Further detailed studies, with more genetic data, are necessary to determine the correct placement of the taxon.

4.2. Non-monophyly of nominal species

Recent molecular studies of *Ochotona* have provided important insights into relationships within the genus. They have also provided a large number of conflicting sequences that indicate a need for future phylogenetic assessments and taxonomic delimitation. In one-third of the cases where multiple sequences were available for a nominal species, that species was not recovered as monophyletic. There are multiple possible reasons for the non-monophyly of these taxa, including specimen misidentification and/or mtDNA introgression between species. This highlights the need to associate

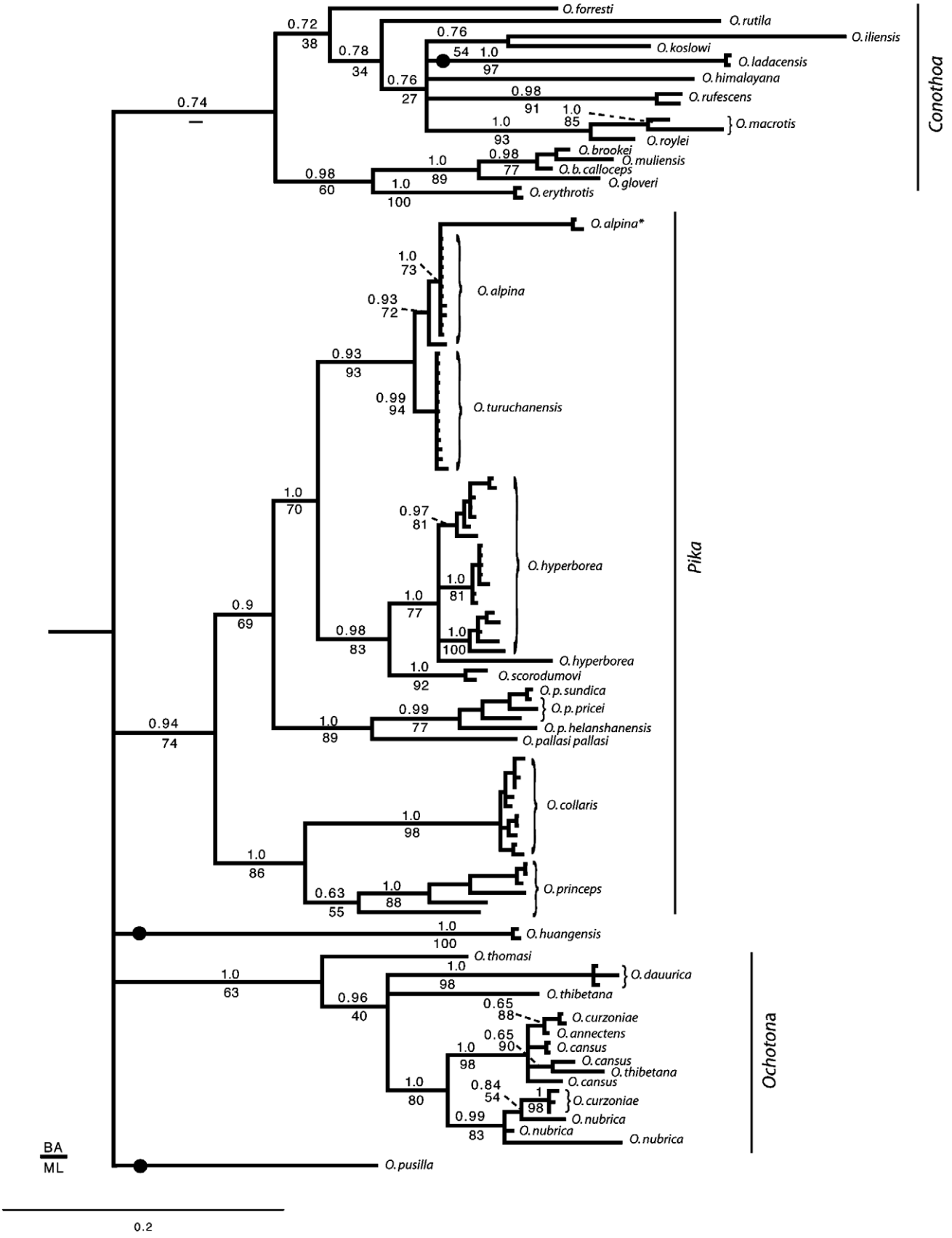

Fig. 1. Consensus tree obtained from phylogenetic analysis of cyt *b* sequences using MrBayes. Nodal support indicated by Bayesian posterior probabilities (BA, top) and ML bootstrap values, (ML, bottom). Support not shown for terminal nodes between conspecific taxa. Subgeneric assignments from Hoffmann and Smith (2005) are shown where subgenera are monophyletic. Black dots indicate variable position of root (see text).

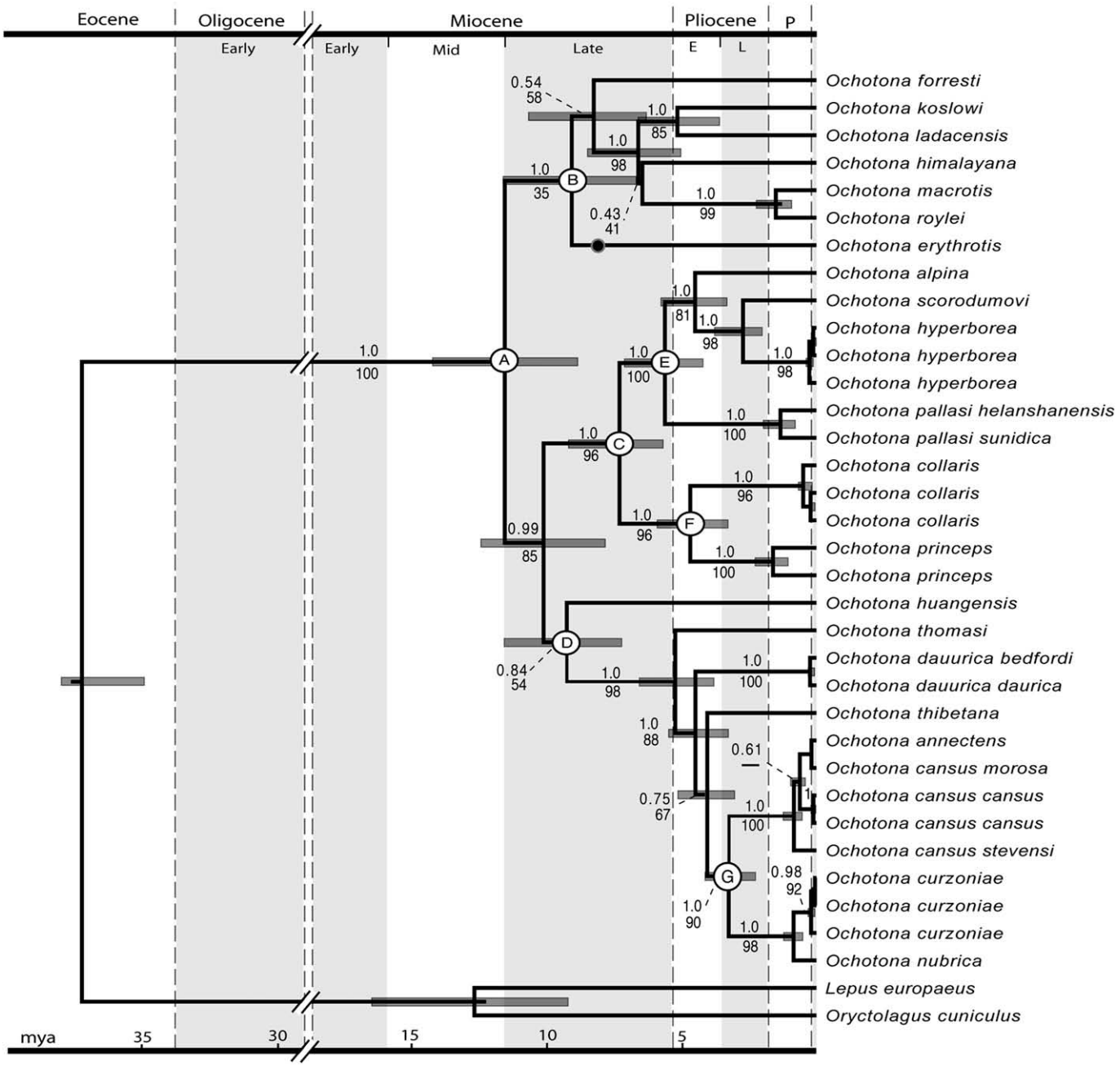

Fig. 2. Divergence dates and 95% credibility intervals resulting from analysis of the 37 mya rooted model (cyt *b* + ND4 dataset) in Bayesian relaxed molecular dating technique implemented in BEAST. Nodal support as in Fig. 1. Geological time scale follows Gradstein et al. (2004).

Table 3
Estimated tMRCA for the major splits within *Ochotona*.

	Node[a]	31 Mya Model[b]	37 Mya Model[b]	65 Mya Model[b]
	Root (posterior)	31.641 (29.632–33.527)	36.958 (34.952–38.879)	64.969 (63.03–66.941)
A	*Ochotona*	9.913 (7.701–12.338)	11.626 (8.98–14.317)	20.253 (16.013–24.857)
B	*Conothoa* 'mountain'	7.722 (5.703–9.913)	9.091 (6.842–11.687)	15.971 (11.945–20.221)
C	*Pika* 'northern'	6.246 (4.73–7.77)	7.326 (5.63–9.208)	12.775 (9.806–15.717)
D	*Ochotona* 'shrub–steppe'	7.9878 (6.0845–9.897)	9.3493 (7.2343–11.653)	16.277 (12.765–20.2594)
E	Palearctic	4.7774 (3.6534–6.0814)	9.0886 (6.7772–11.6095)	9.7818 (7.3517–12.1717)
F	Nearctic	3.941 (2.847–5.14)	4.621 (3.345–5.984)	8.045 (5.998–10.329)

[a] Letters in first column correspond to nodes indicated in Fig. 2.
[b] Dates given in millions of years before present, with mean above and 95% Highest Posterior Density (HPD) below.

sequences with voucher specimens that have stable museum catalog numbers, both in papers and in GenBank submissions (Ruedas et al., 2000; Peterson et al., 2007). Comprehensive molecular and morphological analyses need to be done to further delimit species within *Ochotona*. This also suggests that until species can be better delimited within the group, comparative phylogenetic analyses

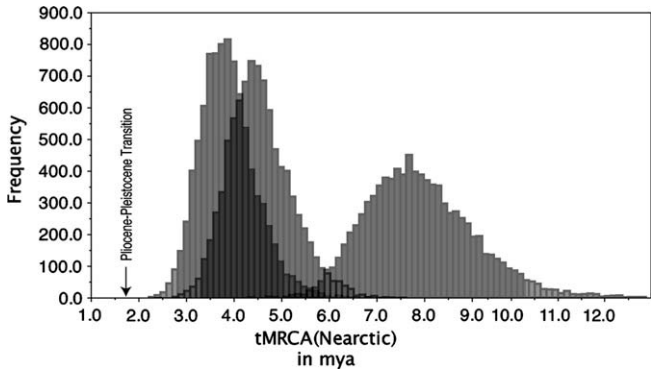

Fig. 3. Range of posterior estimates for tMRCA (time to most recent common ancestor) of Nearctic pikas; divergence dates estimated under the 31, 37, and 65 mya root models (left to right).

need to verify both morphological species identity and mtDNA haplotypes for their results to be repeatable and easily interpreted. Recent studies (Yang et al., 2008) have provided interesting and compelling evidence of adaptive evolution at the protein level within *Ochotona*, and better phylogenetic resolution would almost certainly allow researchers to address and rigorously test more complex hypotheses.

The Northern pika (*O. hyperborea*) and the American pika (*O. princeps*) have long been considered to be two of the most morphologically variable pika species (Smith et al., 1990; Smith and Weston, 1990; Lissovsky, 2003), with 11 and 36 subspecies described, respectively. On a molecular basis, these two species are also some of the most divergent, with intraspecific levels of divergence comparable to or greater than those observed between other species. This coincides with Hafner and Sullivan's (1995) findings of high degrees of genetic differentiation between populations of *O. princeps* resulting from a history of population fragmentation. The incorporation of additional samples of *O. hyperborea* increased the support for the main geographic clades of *O. hyperborea* described by Lissovsky et al. (2007). In contrast, relatively little genetic divergence is evident in *O. collaris*, suggesting a recent population expansion in this species, similar to that inferred for *O. turuchanensis*. While the latitudinal contrasts in climate and glaciation may explain the differences between geographic structuring in *O. collaris* and *O. princeps* (Hafner, 1994), it does less to explain the differences between *O. hyperborea* and *O. collaris*.

4.3. Nearctic pikas diverged before the Pleistocene

Monophyly of the North American pikas was recovered in all phylogenetic analyses. While we are not the first to recover this relationship, our inclusion of multiple samples from throughout the range of both *O. hyperborea* and *O. collaris* makes this the most rigorous test of Nearctic pika monophyly to date. As the addition of multiple intraspecific samples results in non-monophyly for a number of other species of pikas, we felt that this represents significant and meaningful support for the monophyly of the North American taxa. Given the large number of Pleistocene trans-Beringian exchanges observed in other mammalian taxa (Steppan et al., 1999; Galbreath and Cook, 2004; Waltari et al., 2007), the inclusion of these additional individuals is necessary to test the monophyly of the extant North American species. Unfortunately, because *O. collaris* and *O. princeps* are reciprocally monophyletic, these data tell us nothing about the direction of colonization (i.e., whether *O. collaris* represents a separate northern invasion from a southern Nearctic ancestor shared with *O. princeps*).

The oldest record of North American *Ochotona* is *O. spanglei*, known from the Miocene in Oregon (Shotwell, 1956). Fossil evidence has been used to suggest that *O. collaris* and *O. princeps* shared a common ancestor that migrated over from Asia in the early Pleistocene or late Pliocene, representing a second or third trans-Beringian dispersal event for pikas (Erbajeva, 1994), and diverged during the Pleistocene (Guthrie, 1973). *Ochotona whartoni* and a smaller species of pika (presumably *O. collaris* or *O. princeps*) were present in the same sites in the Yukon Territory during the Pleistocene (Harington, 1978; Weston, 1981) at least as early as the Sangamon interglacial (0.130–0.115 mya, Marine Isotope Stage 5e). Our data support an earlier divergence between the Nearctic and Palearctic lineages, as well as an earlier split between the two lineages of extant Nearctic species (*contra* Guthrie, 1973; Niu et al., 2004). While we cannot exclude the possibility that the lineages that became to *O. collaris* and *O. princeps* diverged in Asia and dispersed to North America separately, the most biogeographically parsimonious explanation is that one common ancestor crossed the Bering Land Bridge and subsequently diverged.

It is generally suggested that *Ochotona* invaded North America twice, once by *O. spanglei* and once by the *O. princeps–collaris* lineage. All three scenarios (31, 37, and 65 mya roots) indicate a sufficiently old Nearctic split as to accommodate an *O. spanglei* ancestor. Our results suggest that the Nearctic pikas shared a most recent common ancestor with the Palearctic clade in the subgenus *Pika* between 4.7 and 15.7 million years ago (depending on the calibration of the leporid–ochotonid split).

4.4. Relaxed molecular clocks and speciation in Ochotona

Despite the broad swath of time represented by each model, there are some biogeographic and evolutionary conclusions that can be drawn from our results. The middle Miocene to early Pliocene was considered to be the 'acme of development' for pikas (Dawson, 1967; Erbajeva, 1994), and most of the basal splits on our calibrated trees fall within this period. *Ochotona pusilla* is considered to be a late Pliocene relic based on dental morphology, karyotype, and a relatively old fossil history (Erbajeva, 1994). If the weakly supported rooting of the tree on *O. pusilla* is correct, this species would have shared a common ancestor with the other *Ochotona* at some point prior to the date estimated for the root (Table 3; Fig. 2, Node A). The antiquity of this lineage is supported by fossil *O. pusilla* dating to the Pliocene (Table 1).

The subgenera *Conothoa* and *Pika* speciated quickly, probably during the late Miocene to early Pliocene, and represent some of the oldest surviving lineages within the genus. This contrasts sharply with our findings for the subgenus *Ochotona*, where most speciation events date to the Pliocene and Pleistocene. This may be related to their differing ecotypes, as both *Conothoa* and *Pika* are primarily composed of talus-dwelling species. The low fecundity and low dispersal capability of talus-dwelling pikas may predispose them to vicariant speciation with little competition between incipient species. Species in the subgenus *Ochotona* are mostly burrowing (Smith et al., 1990). Burrowing pikas tend to have larger, more frequent litters and undergo greater population fluctuations (Smith, 1988). Their evolutionary history may have therefore been more influenced by rapid replacement between competing lineages, resulting in fewer surviving lineages relative to talus-dwelling species.

Perhaps the most novel contribution of this study is the application of rigorous hypothesis testing to evaluate alternative scenarios of diversification within a genus. Strict application of molecular clocks, particularly those using externally calibrated rates, can lead to overly narrow, and frequently incorrect, point estimates of divergence dates (Pulquério and Nichols, 2007; Ho, 2007; Fig. 4). Even given the advantages offered by relaxed molecular clock techniques, incorrect fossil calibrations can still result in incorrect dates (Benton and Donoghue, 2007; Fig. 3). However, even lacking a series of well-supported fossil calibrations, specific hypotheses may still be testable. We recovered no support for a Pleistocene

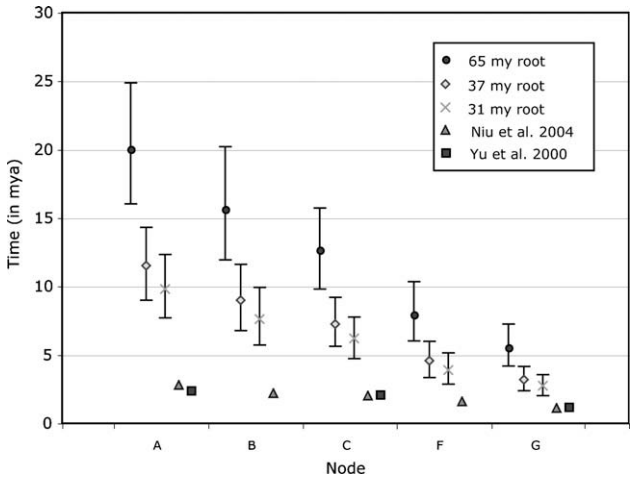

Fig. 4. Comparison of nodal dates estimated under this study with those reported by Yu et al. (2000) and Niu et al. (2004). Nodes correspond to those shown in Fig. 2 and Table 3. Dates resulting from other studies are aligned with columns to which they correspond, but horizontally spaced to show 95% credibility intervals.

common ancestor for Nearctic pikas. Similarly, none of the divergences estimated from strict molecular clock approaches were supported under any of our models (Fig. 4). While the broad sets of credibility intervals for each node encompass a large span of time, and are thus more difficult to correlate to particular geologic and climatic events, they may be more representative of biological reality and the tempo of speciation.

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Appendix A

GenBank sequences used in this study. Specimens with both cyt *b* and ND4 sequence available were used in relaxed molecular clock analyses.

Taxon	Cyt *b*	Length (bp)	ND4	Length (bp)	Citation
O. alpina	AF273009	1140	AF273130	1383	Yu et al. (2000)
	AY056605	402			Niu et al. (2004)
	DQ335482	719			Lissovsky et al. (2007)
	DQ335487	719			Lissovsky et al. (2007)
	DQ335508	674			Lissovsky et al. (2007)
	DQ335509	719			Lissovsky et al. (2007)
	DQ335510	719			Lissovsky et al. (2007)
	DQ335511	719			Lissovsky et al. (2007)
	DQ335512	719			Lissovsky et al. (2007)
	DQ335513	719			Lissovsky et al. (2007)
	DQ335514	719			Lissovsky et al. (2007)
	DQ335515	719			Lissovsky et al. (2007)
	DQ335516	719			Lissovsky et al. (2007)
	DQ335517	719			Lissovsky et al. (2007)
O. annectens	AF273008	1140	AF273129	1383	Yu et al. (2000)
O. brookei	AY056600	402			Niu et al. (2004)
O. b. calloceps	AY191825	402			Niu et al. (2004)
O. cansus cansus	AF273003	1140	AF273125	1383	Yu et al. (2000)
	AF273006	1140	AF273126	1383	Yu et al. (2000)
O. c. morosa	AF273007	1140	AF273127	1383	Yu et al. (2000)
O. c. stevensi	AF273005	1140	AF273128	1383	Yu et al. (2000)
O. collaris	AF176578	682			Hafner et al. (unpublished)
	AF348080	1140	AF348080	1383	Lin et al. (2002)
	AY056608	402			Niu et al. (2004)

(*continued on next page*)

Appendix A. (*continued*)

Taxon	Cyt *b*	Length (bp)	ND4	Length (bp)	Citation
O. curzoniae	AF176581	682			Hafner et al. (unpublished)
	AF273001	1140	AF273122	1383	Yu et al. (2000)
	AF273002	1140	AF273123	1383	Yu et al. (2000)
	AF273004	1140	AF273124	1383	Yu et al. (2000)
	AF432908	402			Niu et al. (2004)
O. dauurica	DQ335519	680			Lissovsky et al. (2007)
O. d. bedfordi	AF273000	1140	AF273134	1216	Yu et al. (2000)
O. d. dauurica	AF273011	1140	AF273135	1190	Yu et al. (2000)
O. erythrotis	AF272999	1140	AF273121	1383	Yu et al. (2000)
	AY056606	402			Niu et al. (2004)
O. forresti	AF272998	1140	AF273120	1383	Yu et al. (2000)
O. gloveri	AY056602	402			Niu et al. (2004)
O. himalayana	AF272997	1140	AF273119	1383	Yu et al. (2000)
O. huangensis	AF272995	1140	AF273117	1383	Yu et al. (2000)
O. h. xunhuaensis	AY191821	402			Niu et al. (2004)
O. hyperborea	AB053257	1140			Takaki et al. (unpublished)
	AF176582	682			Hafner et al. (unpublished)
	AY056603	402			Niu et al. (2004)
	DQ335483	674			Lissovsky et al. (2007)
	DQ335484	719			Lissovsky et al. (2007)
	DQ335485	719			Lissovsky et al. (2007)
	DQ335486	719			Lissovsky et al. (2007)
	DQ335489	719			Lissovsky et al. (2007)
	DQ335492	719			Lissovsky et al. (2007)
	DQ335498	719			Lissovsky et al. (2007)
	DQ335499	719			Lissovsky et al. (2007)
	DQ335500	385			Lissovsky et al. (2007)
	DQ335502	719			Lissovsky et al. (2007)
	DQ335503	719			Lissovsky et al. (2007)
	DQ335504	719			Lissovsky et al. (2007)
	DQ335523	325			Lissovsky et al. (2007)
	DQ335524	344			Lissovsky et al. (2007)
O. iliensis	AY191824	402			Niu et al. (2004)
O. koslowi	AF272993	1140	AF273116	1383	Yu et al. (2000)
O. ladacensis	AF272992	1140	AF273114	1383	Yu et al. (2000)
	AY056609	402			Niu et al. (2004)
O. macrotis	AF273010	1140	AF273133	820	Yu et al. (2000)
O. m. macrotis	AY191820	402			Niu et al. (2004)
O. muliensis	AF421884	402			Niu et al. (2004)
O. nubrica	AF272991	1140	AF273113	1383	Yu et al. (2000)
O. n. lama	AY191823	402			Niu et al. (2004)
O. n. lhasaensis	AY191822	402			Niu et al. (2004)
O. pallasi pallasi	DQ335522	317			Lissovsky et al. (2007)
O. p. helanshanesis[a]	AF272996	1140	AF273118	1383	Yu et al. (2000)
O. p. pricei	AY117696	402			Niu et al. (2004)
	AY056607	402			Niu et al. (2004)
	DQ335520	391			Lissovsky et al. (2007)
O. p. sunidica	AF272990	1140	AF273132	1218	Yu et al. (2000)
O. princeps	AF176579	682			Hafner et al. (unpublished)
	AJ537415	1140	AJ537415	1383	Gissi and Pesole (unpublished)
	AY056604	402			Niu et al. (2004)
	AY292716	1140			Matthee et al. (2004)
	U58940	653			Halanych and Robinson (1999)
	AF272989	1140	AF273112	1383	Yu et al. (2000)
O. pusilla	AY260744	402			Niu et al. (2004)
O. roylei	AF272988	1140	AF273131	1216	Yu et al. (2000)
O. rufescens	AJ132206	1140			Barome et al. (unpublished)
O. rutila	AF515733	402			Niu et al. (2004)
	AY056601	402			Niu et al. (2004)

O. scorodumovi[b]	AF272994[c]	1140	AF273115[c]	1383	Yu et al. (2000)
	DQ335518	683			Lissovsky et al. (2007)
O. thibetana	AF176580	682			Hafner et al. (unpublished)
	AF272986	1140	AF273110	1383	Yu et al. (2000)
O. thomasi	AF272987	1140	AF273111	1383	Yu et al. (2000)
O. turuchanensis	DQ335507	675			Lissovsky et al. (2007)
	DQ335488	719			Lissovsky et al. (2007)
	DQ335490	719			Lissovsky et al. (2007)
	DQ335491	391			Lissovsky et al. (2007)
	DQ335493	719			Lissovsky et al. (2007)
	DQ335494	719			Lissovsky et al. (2007)
	DQ335495	719			Lissovsky et al. (2007)
	DQ335496	694			Lissovsky et al. (2007)
	DQ335497	719			Lissovsky et al. (2007)
	DQ335501	719			Lissovsky et al. (2007)
	DQ335505	479			Lissovsky et al. (2007)
	DQ335506	391			Lissovsky et al. (2007)
	DQ335525	391			Lissovsky et al. (2007)
Lepus europaeus	AJ421471	1140	AJ421471	1383	
Oryctolagus cuniculus	AJ001588	1140	AJ001588	1383	

[a] Considered to be *O. argentata* by Erbajeva and Ma (2006) on the basis of karyotype, pelage, and size.
[b] Although *O. scorodumovi* may be synonymous with *O. mantchurica*, we follow the taxonomy used by Lissovsky et al. (2007) to facilitate comparisons or our results with theirs.
[c] Originally designated as *O. hyperborea* by Yu et al. (2000). We follow the taxonomy of Lissovsky et al. (2007).

Appendix B. Voucher specimens

University of Alaska Museum (UAM) catalog numbers and GenBank Accession numbers for specimens sequenced in this study, arranged alphabetically and by catalog number.

Species	Voucher	GenBank Accession No.	Locality
O. collaris	UAM 31645	EU549741[a]	United States: Alaska; Snowhawk Lake
	UAM 35126	EU549747[a]	
		EU549742[b]	United States: Alaska; White Pass
	UAM 57694[c]	EU549748[a]	United States: Alaska; Pocket Creek
		EU549743[b]	
	UAM 58205	EU549749[a]	United States: Alaska; mountainside NW of headwater lake of Crescent Creek
		EU549738[b]	
	UAM 58445	EU549750[a]	United States: Alaska; E slope of Mt. Kathryn, S of Woodchopper Creek
		EU549739[b]	
	UAM 63935	EU549751[a]	United States: Alaska; Eagle Summit, E side of Steese Hwy on talus slope
		EU549736[b]	
	UAM 63936[c]	EU549752[a]	United States: Alaska; Eagle Summit, E side of Steese Hwy on talus slope
		EU549740[b]	
	UAM 71652[c]	EU549753[a]	Canada: Yukon Territory; Thandlat
		EU549737[b]	
O. hyperborea	UAM 23239[c]	EU549754[a]	Russia: Magadanskaya oblast; Stokovo
		EU549744[b]	
	UAM 80090[c]	EU549755[a]	Russia: Magadanskaya oblast; Contact Station, 150 km NW Ust-Omchug
		EU549746[b]	
	UAM 80812[c]	EU549756[a]	Russia: Magadanskaya oblast; 40 km W Magadan
		EU549745[b]	

[a] Cyt b.
[b] ND4.
[c] Used in molecular dating analysis.

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