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Author(s) :Scott J. Steppan, G. J. Kenagy, Christopher Zawadzki, Rafael Robles, Elena A. Lyapunova, and Robert S. Hoffmann

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Molecular data resolve placement of the Olympic marmot and estimate dates of trans-Beringian interchange

Scott J. Steppan,* G. J. Kenagy, Christopher Zawadzki, Rafael Robles, Elena A. Lyapunova, and Robert S. Hoffmann

Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, USA (SJS, CZ, RR)
Burke Museum and Department of Biology, University of Washington, Seattle, WA 98195, USA (GJK)
Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia (EAL)
Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA (RSH)

* *Correspondent: steppan@bio.fsu.edu*

We reinvestigated the phylogeny of all 15 species of *Marmota* to resolve a conflict between 2 published analyses, one by Kruckenhauser et al. and another by Steppan et al., regarding the Olympic marmot (*M. olympus*) and to improve resolution in the genus. We acquired fresh samples of *M. olympus*, combined all available data on mitochondrial DNA (cytochrome *b* [*Cytb*] and *ND3*/*ND4*), new sequences for *ND3*/*ND4*, and 2,000 base pairs (bp) of the nuclear *RAG1* gene. All analyses indicate that *M. olympus* is a much older, rather than more recent, offshoot of the widespread hoary marmot (*M. caligata*) or Vancouver Island marmot (*M. vancouverensis*). The mitochondrial data and some *RAG1* results are largely congruent, but *RAG1* differs on several points, including: the subgenus *Marmota* appears paraphyletic to *Petromarmota*, with reciprocally monophyletic Palearctic and Nearctic clades; and the long-tailed marmot (*M. caudata*) and Menzbier's marmot (*M. menzbieri*) are not sister species, suggesting mitochondrial introgression. Asia was colonized by *Marmota* from North America at approximately 4.6 million years ago (mya), followed by rapid diversification of several major lineages. *M. olympus* diverged from the *M. caligata–M. vancouverensis* lineage at approximately 2.6 mya, whereas *M. vancouverensis* and *M. caligata* diverged at only about 0.4–1.2 mya. *M. olympus* might have survived in isolation on the Olympic Peninsula in a nunatak refugium throughout a series of glacial maxima.

Key words: cytochrome *b*, historical biogeography, *Marmota olympus*, phylogeny, *RAG1*

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Marmots (genus *Marmota*) are large, generally social ground squirrels (family Sciuridae, tribe Marmotini) with a Holarctic distribution (Fig. 1). The genus currently includes 15 species, and various of these have been the subjects of conspicuous comparative studies of sociality and life history (Blumstein 2007; Ozgul et al. 2007), hibernation physiology (Arnold 1990), and morphological evolution (Cardini et al. 2005, 2007, 2009; Caumul and Polly 2005; Polly 2003). These comparative interests led to 2 recent phylogenetic studies of the genus (Kruckenhauser et al. 1999; Steppan et al. 1999), both of which used the complete mitochondrial gene cytochrome *b* (*Cytb*). Kruckenhauser et al. (1999) also included mitochondrial *ND4* data for a subset of species. The 2 studies produced nearly identical results, with one notable exception, the position of the Olympic marmot (*M. olympus*). This species, consisting of a small and geographically restricted population, is endemic to the mountains of the Olympic Peninsula of Washington (Edelman 2003). The analysis by Kruckenhauser et al. (1999) found *M. olympus* to be a sister species of the Vancouver Island marmot (*M. vancouverensis*), an endangered species endemic to Vancouver Island and separated from the Olympic Peninsula to the south by waters of the Strait of Juan de Fuca (Fig. 1). In contrast, Steppan et al. (1999) found *M. olympus* to be a more basal member of the North American clade *Petromarmota.* The result of Kruckenhauser et al. (1999) suggested that *M. olympus* is a recently diverged population of *M. vancouverensis* or of the parapatric and much more widely distributed hoary marmot (*M. caligata*).

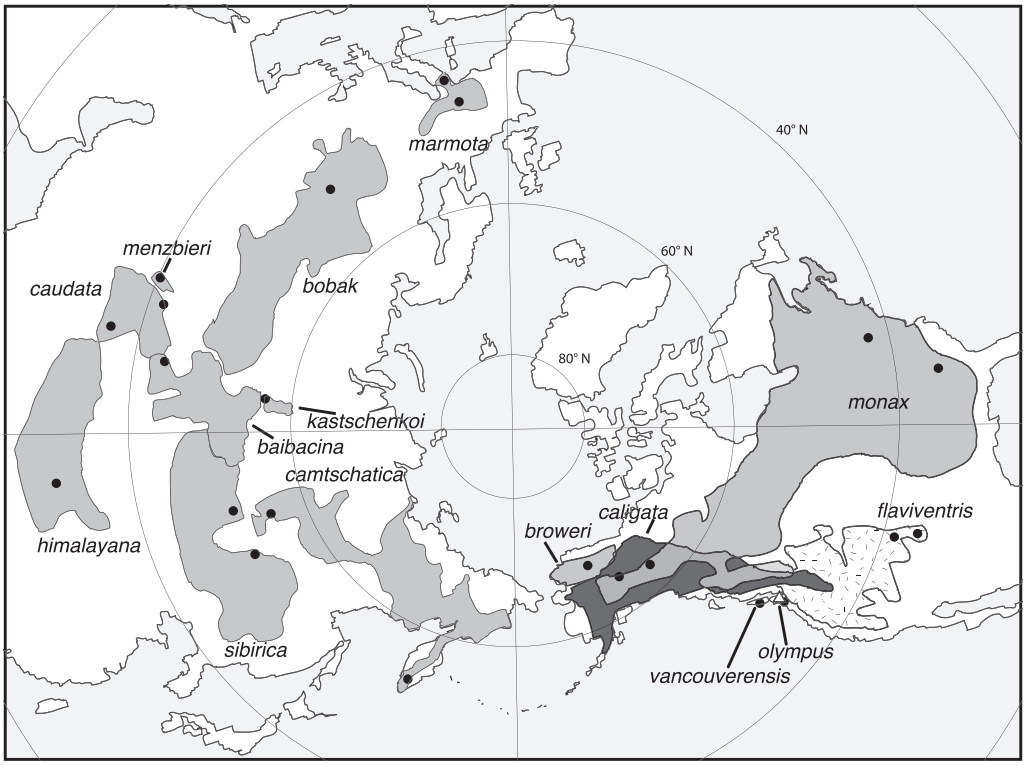

Fig. 1.—Polar-view map showing Holarctic distribution of the genus *Marmota*. Fifteen currently recognized species are labeled, and dots represent the locations of the samples sequenced for this study. Stippling (*M. flaviventris*) and darkest shading (*M. caligata*) are used to distinguish 2 species with large, overlapping ranges in western North America.

Further, if *M. olympus* is specifically distinct, as suggested by morphology (Cardini 2003, 2004), then its sibling relationship to *M. vancouverensis* would represent a case of rapid diversification among all 3 species because genetic distances among them are very low and less than those among most other species of marmot (Kruckenhauser et al. 1999; Steppan et al. 1999). If the result of Steppan et al. (1999) is correct, speciation and divergence were not rapid, and the date of isolation of *M. olympus* from other *Petromarmota* would be much earlier. A subsequent study of short, interspersed elements (SINEs) lacked data for *M. olympus* (Brandler et al. 2010) and so could not resolve this issue.

Steppan et al. (1999) examined the data presented by Kruckenhauser et al. (1999) and suggested that the *olympus* sequence in Kruckenhauser et al. (1999) contained a contaminant. Neither study had access to fresh tissue from *olympus*, and both relied on DNA extracted from the skin (Kruckenhauser et al. 1999) or from skull scrapings (Steppan et al. 1999) of museum specimens. Both marmot phylogenies have been cited together repeatedly (e.g., Brandler and Lyapunova 2009; Cardini 2004; Cardini et al. 2009; Cardini and O'Higgins 2005; Kruckenhauser et al. 2009; Nagorsen and Cardini 2009), but some studies (Herron et al. 2004) have cited only Kruckenhauser et al. (1999) and others (Cardini et al. 2009) only Steppan et al. (1999). Because of the conflicting results in the original papers and in the subsequent citations and because conservation issues related to both *M. vancouverensis* and *M. olympus* require a more accurate understanding of the historical and phylogenetic relationship between *M. olympus* and *M. vancouverensis* and their further relationship to *M. caligata*, we sought to resolve this question by acquiring and sequencing fresh tissues of *M. olympus* and reanalyzing the phylogeny of the genus.

Here we report the new phylogenetic analysis based on additional sequences from 2 individual *M. olympus*. We also sequenced the gene region including portions of transfer RNA_{GLY} ($tRNA_{GLY}$), *ND3*, $tRNA_{ARG}$, *ND4L*, and *ND4* for the individuals included by Steppan et al. (1999) so as to overlap the *ND4* data of Kruckenhauser et al. (1999). We also sequenced 2,147 base pairs (bp) of the nuclear *RAG1* gene to complement the SINE results of Brandler et al. (2010) and allow a concatenation and integration of all available data. This effort expanded the number of species with *ND4* data from 6 to 12, including *M. olympus*. By combining the sets of data we also were able to test species boundaries and improve the survey of intraspecific genetic variation to place the divergences of *M. vancouverensis* and *M. olympus* in context. We further used relaxed molecular-clock dating to estimate divergence dates within the genus and improve estimation of dates for historical trans-Beringian interchange within the genus *Marmota*.

Materials and Methods

Materials and molecular techniques.—The DNA from liver tissue of 2 specimens accessioned of *M. olympus* by the Burke Museum, University of Washington (UWBM), in 2002 was

extracted by standard proteinase K–phenol–chloroform procedures (Maniatis et al. 1982). The entire *Cytb* gene was amplified with primers and protocols outlined by Steppan et al. (1999). The *ND3*/*ND4* region was amplified for most marmots with primers and conditions from Engel et al. (1998). This region overlapped the sequences of Kruckenhauser et al. (1999) by an average of 609 nucleotides. Several species also were amplified with the primers and conditions from Kruckenhauser et al. (1999) and resulted in complete overlap (1,223 bp). The nuclear gene *RAG1* was sequenced for 1 individual per species. Polymerase chain reaction amplification was achieved using the combination of rodent specific primers S70, S73 and S77, and S71, with conditions described in Steppan et al. (2004). A fragment of 2,144 bp, including the faster evolving divergent region (approximately the first 1,000 bp), was sequenced using the amplification and internal primers S105, S102 (CATCTGCCTCACTGCCCAYC), S118, S119, and S104 (Steppan et al. 2004). We were unable to obtain the complete *RAG1* fragment for all species of *Marmota*. Therefore, incomplete sequences were used for the Himalayan marmot (*Marmota himalayana*; 1,997 bp), *M. olympus* (1,743 bp), *M. marmota* (1,223 bp), and *M. vancouverensis* (502 bp). Published outgroup sequences for *Cytb* (Harrison et al. 2003; Thomas and Martin 1993) consisted of the eastern gray squirrel (*Sciurus carolinensis*), yellow ground squirrel (*Spermophilus fulvus*; AF157908), thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*), long-tailed ground squirrel (*Urocitellus undulatus*; AF157906), Richardson's ground squirrel (*U. richardsonii*), Columbian ground squirrel (*U. columbianus*), California ground squirrel (*Otospermophilus beecheyi*; AF157919), golden-mantled ground squirrel (*Callospermophilus lateralis*), and Cascade golden-mantled ground squirrel (*C. saturates*—Steppan et al. 1999). *RAG1* outgroup sequences were from Père David's rock squirrel (*Sciurotamias davidianus*) and Siberian chipmunk (*Tamias sibiricus*—Steppan et al. 2004). New DNA sequences were deposited with GenBank under accession numbers JF313271–JF313303.

Phylogenetic analyses.—The DNA sequences for each individual were concatenated and aligned by Sequencher 4.2 (GeneCodes, Ann Arbor, Michigan), resulting in a data matrix with 5,316 characters (3,169 mitochondrial and 2,147 nuclear). No manual adjustment was made for such closely related sequences. Phylogenetic analyses were conducted with maximum-likelihood (ML) approaches with PAUP* (Swofford 2002) and Bayesian approaches with MrBayes version 3.12 (Ronquist and Huelsenbeck 2003) and BEAST version 1.4.8 (Drummond and Rambaut 2007). Analyses were conducted on the mitochondrial and nuclear data separately and combined. Maximum-parsimony trees were 1st estimated with PAUP*, and one was randomly chosen for evaluation of models of evolution with Modeltest (Posada and Crandall 1998). For the mitochondrial data the GTR + I + Γ model was chosen by means of the Akaike information criterion (AIC), and parameter values were estimated under this model. ML analyses were conducted with those parameter values with a heuristic search, tree-bisection reconnection, and 20 random-addition replicates. *Callospermophilus* and *Otospermophilus* together form the sister group to *Marmota* in ML analyses of *Cytb* (Harrison et al. 2003; Herron et al. 2004) and thus were elevated to generic status in a review by Helgen et al. (2009). Confidence was assessed by nonparametric bootstrapping (Felsenstein 1985) with 500 replicates, each a heuristic search with 5 random-addition replicates and 5,000-rearrangements limit per replicate. Bayesian analysis of the mitochondrial data with MrBayes used the same model but 4 partitions: the 3 codon positions across all protein coding regions and tRNA.

For the nuclear data the GTR + I model was chosen using AIC, and parameter values were estimated under this model. ML analyses were conducted using those parameter values with a heuristic search, tree-bisection reconnection, and 10 random-addition replicates. Bootstrapping used 100 replicates, each with 10 random-addition replicates and 100,000-rearrangements limit per replicate. Bayesian analysis on the *RAG1* data was performed with MrBayes with and without partitions; in both cases 2 sets of heated chains were run for 10 million generations, saving trees every 1,000 generations. When partitioned by codon, all partitions were unlinked. The combined data placed the *RAG1* sites into a 5th partition. Partition parameters were unlinked. Two sets of 4 heated chains were run for 12 million generations for mitochondrial DNA (mtDNA), 10 million for *RAG1*, and 20 million for combined, and trees were saved every 500 generations for the mitochondrial analysis and every 1,000 generations for the nuclear and combined analyses. Likelihood values and convergence diagnostics visualized by AWTY (Wilgenbusch et al. 2004) indicated that stationarity was achieved well before 5% of chain length, so a 10% burn-in was used for each analysis.

BEAST was used to estimate an ultrametric chronogram for marmots; only the mitochondrial data were used because of the small number of informative sites in *RAG1* and possible gene-tree conflicts between the 2 loci. The data were partitioned as with MrBayes, except that the tRNAs were excluded because the few characters provided little information for accurate parameter estimates that could reduce the accuracy of branch-length estimates. The GTR + I + Γ model was used, with parameters unlinked across partitions. The Markov chain Monte Carlo chain was run for 20 million generations; trees and parameters were saved every 2,000 generations. Two fossil calibrations were used. The root of the tree was set to a mean prior of 33.2 million years ago (mya) with a normal distribution and standard deviation (*SD*) of 1.0, based on the estimate by Mercer and Roth (2003). Their estimate was derived on the assumption that the oldest sciurid was *Douglassciurus*, and they set the root of their tree at 36 mya. They then estimated an ultrametric tree under a molecular clock constraint. We interpolated node ages from their tree figure for the relevant node (the most recent common ancestor of tree squirrels and marmots is more recent than that of all squirrels). We used an interpolation from the estimate of Mercer and Roth (2003) rather than a direct fossil calibration

because the best-understood fossil near the root falls outside the taxonomic sampling here. The 2nd calibration used *Marmota minor* as the oldest marmot fossil at 10.3 mya (J. Alroy, Macquarie University, Sydney, pers. comm.; Paleobiology Database, http://www.paleodb.org, accessed 1 January 2010). We assigned this date to the most recent common ancestor of marmots and their sister group (in this study, the clade of *Callospermophilus lateralis*, *C. saturatus*, and *Otospermophilus beecheyi* plus a cluster of pseudogenes). The point along the branch leading from *Spermophilus* to the most recent common ancestor of crown-group *Marmota* at which the fossil diverged is unknown, and this date is just a minimum age; the actual divergence of *Marmota* from its sister group could be earlier. We therefore assigned a prior mean of 10.3 mya with a normal distribution and *SD* of 0.9 mya. This combination yielded a 97.5 percentile of 11.4 mya to coincide with the 95% confidence interval of 1st appearance based on gap sizes in the marmot fossil record as estimated by the Paleobiology Database, with the taxon name ''Marmota.'' In this procedure numerous uncertainties are not fully accounted for (e.g., precise phylogenetic position of the fossils, accuracy of fossil dates, and accuracy of the estimates from Mercer and Roth [2003]), and the absolute dates presented here should be interpreted with caution. Omission of the pseudogenes from the BEAST analysis produced no significant impact on estimated dates or credibility intervals (highest posterior density intervals [*HPD*s]).

Results

Most species and interspecific nodes in the mitochondrial ML phylogeny (Fig. 2) were very robust; only 3 interspecific nodes had <95% ML bootstrap and Bayesian posterior probabilities. The western North American species (yellow-bellied marmot [*M. flaviventris*], *M. caligata*, *M. olympus*, and *M. vancouverensis*) fell together as the subgenus *Petromarmota*. The North American woodchuck (*Marmota monax*) appeared as part of the subgenus *Marmota*. Relationships at the base of the subgenus *Marmota* remain poorly resolved and include 4 lineages: *M. monax*, Alpine marmot (*M. marmota*), the Alaska marmot (*M. broweri*)–long-tailed marmot (*M. caudata*) clade, and the Bobak marmot (*M. bobak*)–Siberian marmot (*M. sibirica*) clade. Bayesian results differed slightly in the resolution of this virtual polytomy, placing *M. monax* and *M. marmota* as sister species and, together, sister to the *M. bobak–M. sibirica* clade, rather than as sequential outgroups within the subgenus as did ML. None of the analyses yielded robust resolution for the base of subgenus *Marmota*.

The new samples of *M. olympus* were grouped with the *Cytb* sequence from Steppan et al. (1999) as the sister group to *M. caligata–M. vancouverensis*, diverging shortly after the basal split with *M. flaviventris*. The sequence of *M. olympus* from Kruckenhauser et al. (1999) was grouped with *M. vancouverensis*. The 2 gene regions *Cytb* and *ND3/ND4*, when analyzed separately, produced congruent trees (results not shown). All species were monophyletic, with 100% ML bootstrap and posterior probabilities (except 98% ML bootstrap and 91% posterior probabilities for *M. flaviventris*, and the paraphyletic *M. vancouverensis*), including *M. caligata* with respect to *M. vancouverensis*, to which *M. caligata* is very similar genetically. Genetic distance (ML) from the Kruckenhauser et al. (1999) *M. olympus* was 0.6–0.7% for *M. vancouverensis* and 2.0–3.0% for *M. caligata*, whereas the new sequences of *M. olympus* differed by 7.4–7.8% from both *M. vancouverensis* and *M. caligata*.

The topology of the Bayesian mtDNA chronogram (Fig. 3) was identical to that of the ML tree (Fig. 2). Several key dates are apparent in the Bayesian chronogram (Fig. 3). Crown-group marmots appear to have begun diversifying around 6 mya (95% *HPD* = 4.6–7.5 mya), and several lineages diverged in rapid succession in subgenus *Marmota*, between 4 and 5 mya. The Nearctic woodchuck *M. monax* was sister to the remaining members of its clade, which is entirely Palearctic except for *M. broweri*, which occurs only in northern Alaska. Given the 95% *HPD*, the Palearctic mtDNA lineage appears to have split from the Nearctic *M. monax* between 3.7 and 6.0 mya. *M. olympus* appears to have diverged from *M. caligata–M. vancouverensis* approximately 2.6 mya (95% *HPD* = 1.7–3.7 mya), whereas *M. caligata* and *M. vancouverensis* mitochondrial lineages diverged much more recently, between 0.4 and 1.2 mya (95% *HPD*). Intraspecific variation was undoubtedly the greatest in *M. flaviventris*; its most recent common ancestor was 2.6 mya (95% *HPD* = 1.6–3.8 mya), comparable to the timing of divergences between other sister groups (e.g., *M. olympus* and *M. caligata* + *M. vancouverensis*; *M. caudata* and Menzbier's marmot [*M. menzbieri*]; *M. bobak* and gray marmot [*M. baibacina*]–forest-steppe marmot [*M. kastschenkoi*]; and *M. siberica* and *M. himalayana*).

The *RAG1* data contained 37 variable characters within *Marmota*, only 6 of which were parsimony informative. Although no individual was heterozygous, most substitutions were autapomorphies, resulting in the few synapomorphies. ML analysis of *RAG1* resulted in 2 equally likely trees (Fig. 4), differing only in the placement of *M. vancouverensis*, the individual with the least amount of sequence data (and from the less-variable 3′ region). These trees also recovered a monophyletic *Marmota* but with far lower support than in Fig. 2 for all nodes. In addition, the basal split differed from that seen in the mitochondrial tree; the Nearctic and Palearctic species formed reciprocally monophyletic groups. *Petromarmota* (*M. caligata*, *M. olympus*, *M. vancouverensis*, and *M. flaviventris*) was monophyletic, with 62% ML bootstrap. Few clades were resolved within these 2 continental clades, and none of them robustly. The 3 clades common to both mtDNA and *RAG1* were *baibacina* + *bobak*, *caligata* + *vancouverensis* + *olympus*, and *Petromarmota*. Notable *RAG1* clades conflicting with the mtDNA tree were the black-capped marmot (*camtschatica*) + *caudata* (67% ML bootstrap) and *himalayana* + *marmota* (9% ML bootstrap).

Bayesian and likelihood analyses of the combined data placed *M. monax* and *M. broweri* as sister species and agreed with the mitochondrial data in placing *M. olympus* outside the *M. caligata–M. vancouverensis* pair. Both analyses yielded

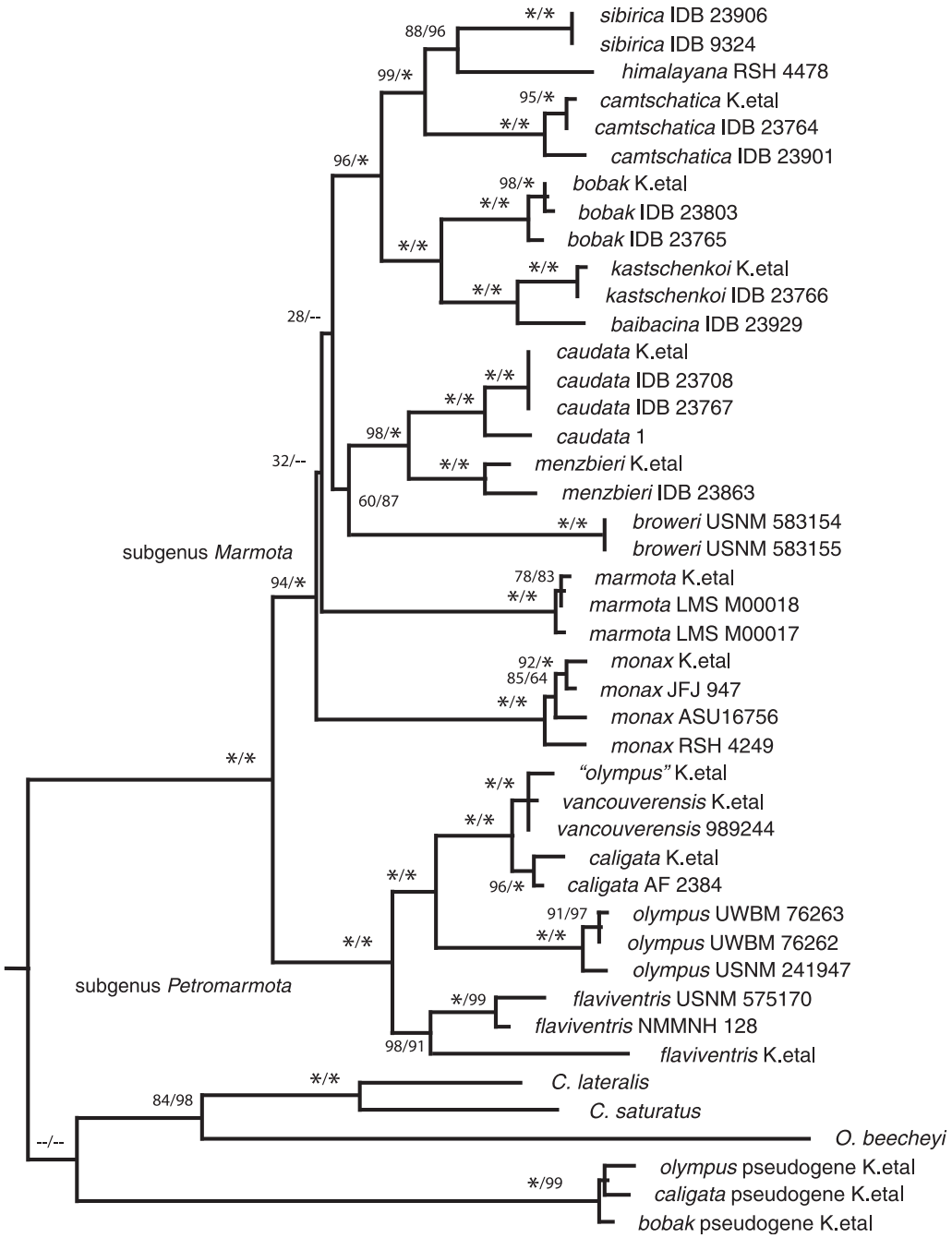

Fig. 2.—Maximum-likelihood (ML) tree of the mitochondrial DNA data for *Marmota*. Specimen identification as in Steppan et al. (1999); sequences from Kruckenhauser et al. (1999) indicated by K.etal. Numbers above branches indicate ML bootstrap values and Bayesian posterior probabilities, in that order, expressed as percentages. An asterisk (*) indicates 100%. Sample identified as ''*olympus*'' K.etal. was originally identified as such but is proposed here to be contaminated. *C.* = *Callospermophilus* and *O.* = *Otospermophilus* for outgroups.

conflicting rooting in the genus; Nearctic and Palearctic clades were monophyletic in the Bayesian analysis (Fig. 5), and subgenera were monophyletic in ML (not shown), which agreed with the mitochondrial trees.

Discussion

Marmota olympus and the M. caligata group.—Examination of our mitochondrial and nuclear data indicates that the Olympic marmot (*M. olympus*) is not most closely related to the Vancouver Island marmot (*M. vancouverensis*), despite their geographic proximity, separated by only a short distance across the Strait of Juan de Fuca (Fig. 1). Instead, *M. olympus* more likely represents a relict of an ancestor of the subgenus *Petromarmota*. Our conclusions conflict with those of Herron et al. (2004), who used the sequence data of Kruckenhauser et al. (1999) for *M. olympus*. Our new results reinforce the suggestion (Steppan et al. 1999) that the DNA sequence of Kruckenhauser et al. (1999) for *M. olympus* might have resulted from a contamination with material from *M.*

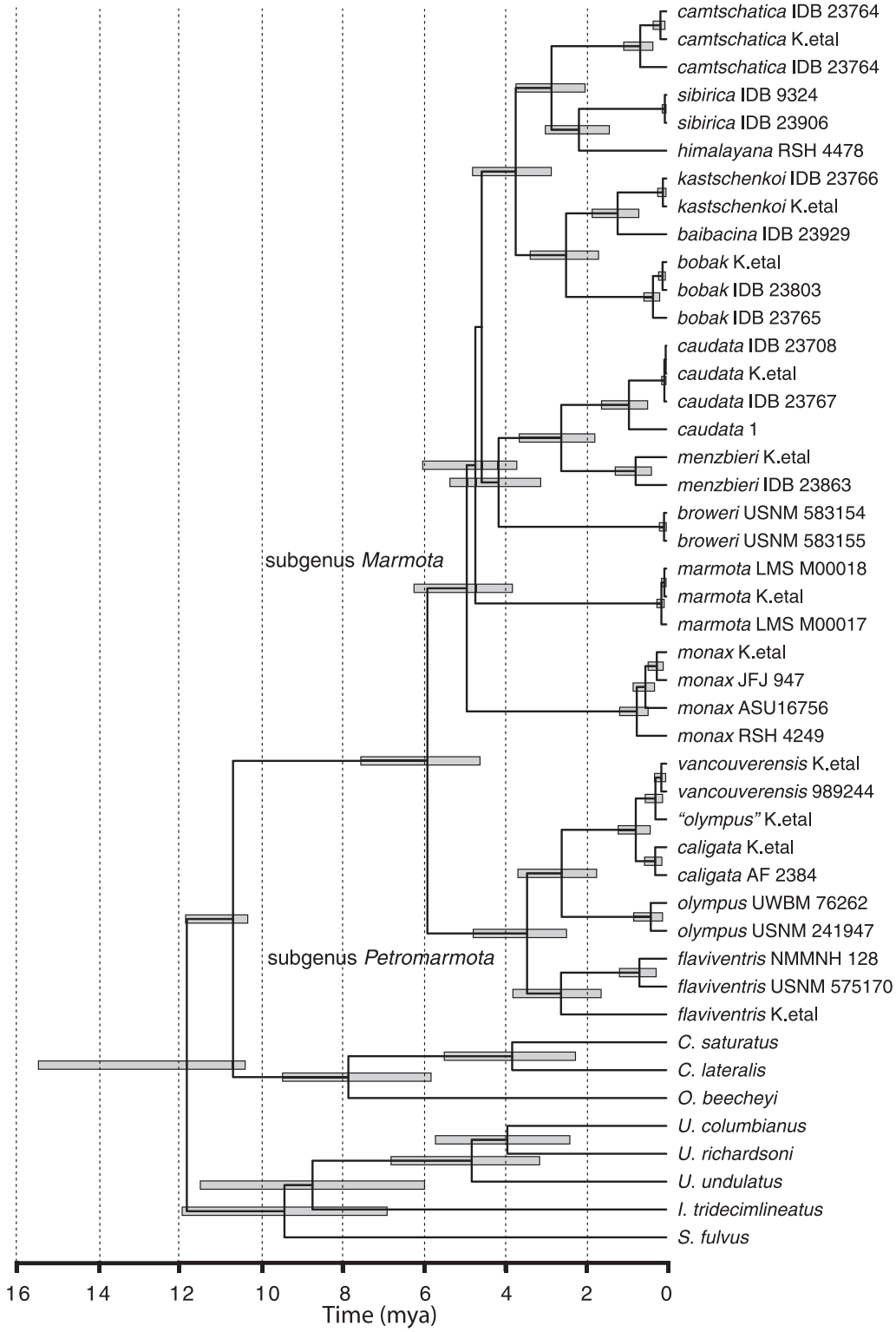

Fig. 3.—Chronogram estimated with BEAST based on mitochondrial DNA data for *Marmota*. Gray bars indicate 95% highest posterior density intervals (*HPD*s). Time scale in millions of years. *C.* = *Callospermophilus*, *O.* = *Otospermophilus*, *U.* = *Urocitellus*, *I.* = *Ictodomys*, and *S.* = *Spermophilus* for outgroups.

vancouverensis. Whereas a 1% sequencing error would account for nonidentity (Steppan et al. 1999), we also cannot exclude the possibility of a recent mitochondrial introgression event as the basis of the placement of *M. olympus* with *M. vancouverensis*. Given the monophyly and short mitochondrial coalescence times for all other species, lineage sorting seems a less likely explanation for the incongruence than either contamination or introgression.

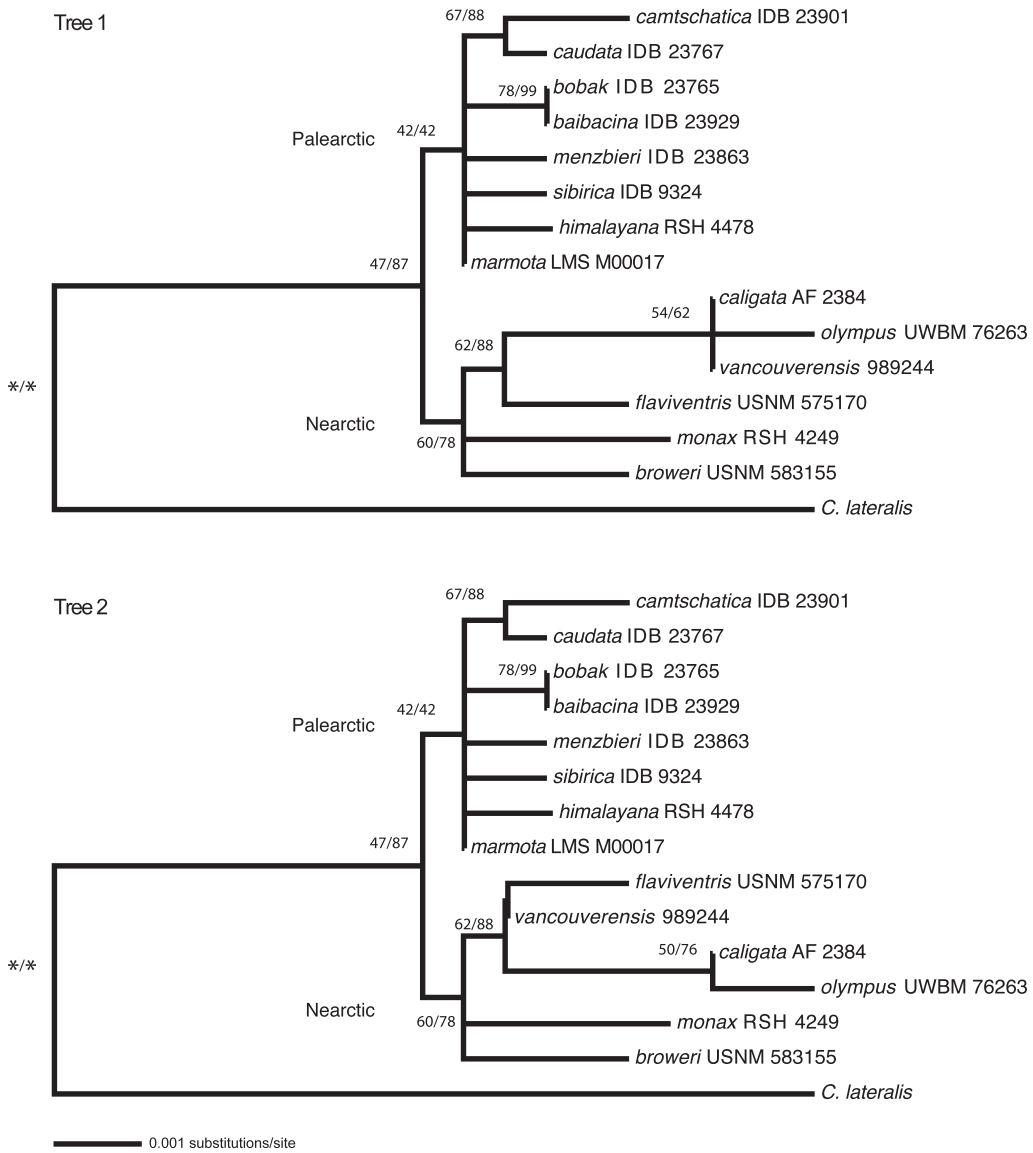

Fig. 4.—Two equally likely maximum-likelihood (ML) trees estimated from the *RAG1* data for *Marmota*. The only topological difference between the 2 trees is the placement of *M. vancouverensis*. Numbers above branches indicate ML bootstrap values and Bayesian posterior probabilities, in that order, expressed as percentages. An asterisk (*) indicates 100%. *C.* = *Callospermophilus* for the outgroup.

Given the strong genetic similarity of *M. vancouverensis* and *M. caligata* and their geographic proximity (*M. caligata* is found just across the narrow Johnstone Strait from Vancouver Island on the mainland of British Columbia, Canada), *M. caligata* seems likely to be paraphyletic with respect to *M. vancouverensis*. If it is, Vancouver Island marmots effectively represent a strongly differentiated population with a high rate of phenotypic divergence (Cardini and O'Higgins 2004). We did not find evidence of paraphyly of *M. caligata*, but our sample contained only 2 specimens of *M. caligata*, 1 from far to the north, in the Alaskan interior, and 1 from the northern Cascades.

Phylogenetics of Marmota.—On a broader phylogenetic scale, the expanded set of mitochondrial data increases our confidence in the phylogenetic relationships revealed by both Kruckenhauser et al. (1999) and Steppan et al. (1999) for this locus. Bootstrap values have increased in our new analysis for nearly every node—most prominently the *M. olympus–M. caligata* clade, from 80% (Steppan et al. 1999) to 100% in the present study, the 6-species *M. bobak–M. sibirica* clade (from 78% to 96%), and *M. himalayana–M. sibirica* (from 75% to 88%). The added data do not robustly support any single resolution of the quadrachotomy of the subgenus *Marmota* over any other, however. All species appear monophyletic.

The addition of nuclear data complicates this picture. As in Brandler et al. (2010), the 2 subgenera of Steppan et al. (1999) are not sister taxa; instead *Petromarmota* is nested inside *Marmota*, whereas the basal split separates Nearctic from Palearctic clades. According to the topology of the nuclear tree, expansion across the Bering land bridge must have happened early in the history of marmots, with the result that no subsequent recrossing would be needed to explain the speciation of *M. broweri*. The *RAG1* data, however, provide much lower support

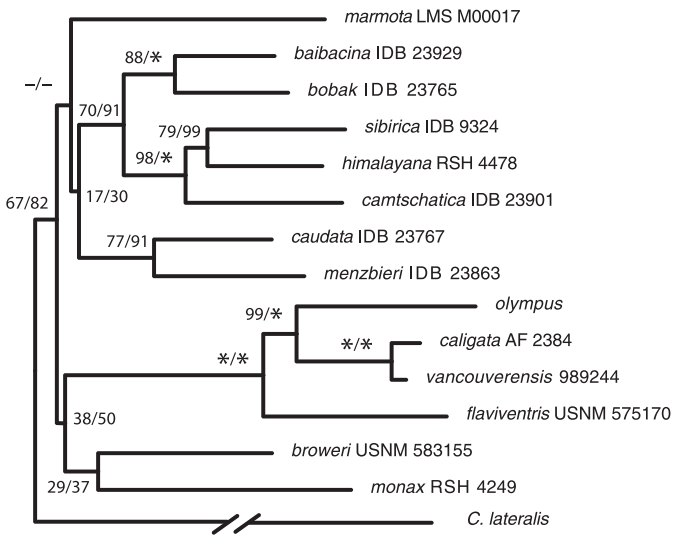

Fig. 5.—Partitioned Bayesian tree estimated from the combined mitochondrial DNA (mtDNA) and nuclear DNA data for *Marmota*. Numbers above branches indicate maximum-likelihood bootstrap values and Bayesian posterior probabilities, in that order, expressed as percentages. An asterisk (*) indicates 100%. *M. olympus* is a concatenation of mtDNA from UWBM 76262 and *RAG1* from UWBM 76263. *C.* = *Callospermophilus* for the outgroup.

values (only 6 parsimony-informative characters), so the incongruence might be explained by phylogenetic error in the nuclear data. Alternatively, the trees could be correct but the gene histories different as a result of differential patterns of lineage sorting. Concatenated sets of data yield 2 different resolutions intermediate between those of the individual loci. By partitioning the nuclear data and estimating parameters for them separately, the Bayesian analysis effectively gave more weight to characters in the slowly evolving *RAG1*, and the tree therefore resembles the *RAG1* tree more than does the unpartitioned ML tree. In both concatenated analyses, however, support values at basal nodes were very low.

Brandler et al. (2010) suggested that the difference between their SINE data and the mtDNA tree regarding *M. caudata* and *M. menzbieri* could be explained by an ancient introgression event, which would have caused those 2 to appear as sister species in the mtDNA tree but not in the nuclear DNA tree. Evidence for such an ancient event is present in another marmotine lineage, *Neotamias* chipmunks (Good et al. 2008). Our *RAG1* results are consistent with the SINE data of Brandler et al. (2010) and allozyme data of Mezhzherin et al. (1999) in not indicating sister species, and if the introgression hypothesis is correct, that event could have taken place as long ago as 3.5 mya (95% maximum estimate). The branches leading to each crown group are approximately 1.7 mya, the most recent common ancestor of the 2 species is estimated at 2.6 mya, and coalescent times within each species are <1 mya. Therefore, introgression could have occurred as much as 1 million years after the coalescence of those 2 mtDNA lineages, provided that ancestral polymorphism had been maintained for a lengthy period. An ancient trans-Beringian hybridization event also could explain the mitochondrial placement of Alaskan *M. broweri* within a Palearctic clade (if the nuclear trees are correct), but in that case, the event probably would have occurred approximately 4–5 mya. In this scenario, *M. broweri* could be sister to *M. monax* (as indicated by nuclear data), and the only trans-Beringian crossing would be that involved with the hybridization. Alternatively, if the hybridization was more recent, then the introgressing Palearctic species must have subsequently gone extinct, leaving its genetic legacy within *M. broweri*. We suggest the more likely possibility of a lack of resolution in the nuclear data combined with lineage sorting; testing this would require a much larger sampling of loci, as proposed by Brandler et al. (2010).

Historical biogeography.—Molecular clock dating allows us to estimate the timing of several key events using the mitochondrial tree, notably the invasion of Asia by marmots approximately 4.6 mya and the isolation of *M. olympus* from its sister clade, the *M. caligata* group (including *M. vancouverensis*), approximately 2.6 mya. Even if the nuclear data are correct regarding the monophyly of continental clades, the short branches in both mitochondrial and nuclear trees at the base of the Palearctic clades indicate that at least 4 extant Palearctic lineages appeared within a period of <1 million years. This pattern suggests a rapid expansion of marmots across Eurasia followed by nearly simultaneous geographic isolation and allopatric or parapatric speciation. And, unlike the situation in western North America, species remain allopatrically distributed (Fig. 1).

Marmota olympus appears to have diverged from the *M. caligata* group (including *M. vancouverensis*) approximately 2.6 mya, whereas *M. vancouverensis* and *M. caligata* diverged only about 0.4–1.2 mya. At the time of its divergence *M. olympus* probably occurred over a broader geographic area of the Pacific Northwest or western North America, perhaps even before completion of the uplift of the Cascade and Olympic montane axes. In the absence of recognizable fossils of *M. olympus* reconstruction of the geographic background of this speciation event is difficult. However, we can comment on the reinforcement and maintenance of this species in its isolated peninsular alpine habitat. Throughout a series of Quaternary glacial maxima, portions of the alpine zone of the Olympic Mountains remained ice-free above lowland sheets of glacial ice and thus provided refugia, in this case known as ''nunataks'' (Easterbrook 1992; Houston et al. 1994), in which species such as *M. olympus* would have survived in isolation. The later date of the divergence of *M. vancouverensis* from *M. caligata* (0.4–1.2 mya) probably can be explained by the history of that island's isolation with more recent connections to the mainland. With our updated phylogeny and a new chronogram of the genus *Marmota*, we have generated and clarified a useful framework for new hypotheses that seek to account for the geographic history and interrelations of western North American marmot species.

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APPENDIX I

List of *Marmota* specimens sequenced. Abbreviations: University of Alaska, Fairbanks (AF); Appalachian State University (ASU); Institute of Developmental Biology, Moscow (IDB); Laboratory of Molecular Systematics, Smithsonian Institution (LMS); New Mexico Museum of Natural History (NMMNH); National Museum of Natural History (USNM); and Burke Museum, University of Washington (UWBM). Tissues collected by J. F. Jacobs (JFJ) and R. S. Hoffmann (RSH) are housed in the collections of the Smithsonian Institution.

Marmota baibacina centrali.—IDB 23929. Kazakhstan, Chimkentsk obl., Bol'shoi Kokpak valley; 41°45′N, 69°30′E.

Marmota bobak.—IDB 23765. Ukraine, Khar'kovsk obl., Velikii, Byrluksk rai.; 50°N, 37°20′E.

Marmota bobak.—IDB 23803. Ukraine, Khar'kovsk obl., Velikii, Byrluksk rai.; 50°N, 37°20′E.

Marmota broweri.—USNM 583154. USA, Alaska, Brooks Range, vicinity Anaktuvuk Pass; 68°10′N, 152°W.

Marmota broweri.—USNM 583155. USA, Alaska, Brooks Range, vicinity Anaktuvuk Pass; 68°10′N, 152°W.

Marmota caligata.—AF 2384. USA, Alaska, vicinity Fairbanks; 65°N, 145°W.

Marmota camtschatica.—IDB 23764. *M. camtschatica camtschatica.* Russia, Kamchatsk obl., Nilkovski rai.; 53°N, 157°30′E.

Marmota camtschatica.—IDB 23901. *M. camtschatica doppelmayri.* Russia, Buryatiya, Severo-Baikalsk rai.,upper Chai River; 55°30′N, 109°E.

Marmota caudata.—1. *M. caudata caudata.* Pakistan, Northern Terr., Hunza, vicinity Khunjerab Pass; 36°50′N, 75°20′E.

Marmota caudata.—IDB 23767. *M. caudata aurea.* Kazakhstan, Dzhambulsk obl., Nerke; 43°N, 71°30′E.

Marmota caudata.—IDB 23708. *M. caudata aurea.* Kazakhstan, Dzhambulsk obl., Nerke; 43°N, 71°30′E.

Marmota flaviventris.—USNM 575170. *M. flaviventris luteola*. USA, Colorado, Gunnison Co., ~7 mi. N of Crested Butte, along East River; 38°53′N, 106°58′W.

Marmota flaviventris.—NMMNH 128. *M. flaviventris obscura*. USA, New Mexico, Taos Co., Sangre De Cristo Mountains, N of Santa Fe; 36°30′N, 105°30′W.

Marmota himalayana.—RSH 4478. *M. himalayana robusta*. China, Qinghai Prov., Yushu Aut. Pref., Nangqen Co., Bei-zha Forestry Sta., Ba Qu (river); 31°45′N, 96°30′E.

Marmota kastschenkoi.—IDB 23766. Russia, Novosibirsk obl., vicinity Novosibirsk; 55°N, 83°E.

Marmota marmota.—LMS M00017. *M. marmota marmota*. Switzerland, Canton Grisons, Davos; 46°47′N, 9°50′E.

Marmota marmota.—LMS M00018. *M. marmota marmota*. Italy, Modena, Monte Cimone; 44°12′N, 10°42′E.

Marmota menzbieri.—IDB 23863. *M. menzbieri zachidovi.* Uzbekistan, Tashkentsk obl., Chatkalsk zapovednik, vic. Parkent; 41°15′N, 70°E.

Marmota monax.—RSH 4249. *M. monax ochracea*. Canada, Yukon, Ethel Lake; 63°21′N, 136°W.

Marmota monax.—JFJ 947. *M. monax rufescens.* USA, New York, Tompkins Co., vicinity Ithaca; 42°30′N, 76°30′W.

Marmota monax.—ASU 16756. *M. monax monax.* USA, North Carolina, no exact locality; 35°30′N, 82°30′W.

Marmota olympus.—USNM 241947. USA, Washington, Quinault River; 49°30′N, 125°W.

Marmota olympus.—UWBM 76262. USA, Washington, second basin west of Hurricane Hill; 47°59′51″N, 123°32′07″W, 5,400 ft.

Marmota olympus.—UWBM 76263. USA, Washington, Wolf Creek trail; 47°58′26″N, 123°30′78″W, 4,800 ft.

Marmota sibirica.—IDB 9324. *M. sibirica sibirica*. Russia, Chitinsk Obl.Ononsk rai., Pobeda; 57°30′N, 116°E.

Marmota sibirica.—IDB 23906. *M. sibirica caliginosus*. Russia, Buryatiya, Selenginsk rai., Toion, Gusinoe Lake; 51°N, 106°15′E.

Marmota vancouverensis.—989244. Canada, British Columbia, Vancouver Island, no exact locality; 49°30′N, 123°30′W. Blood sample collection number 989244 from Andrew Bryant.